8-26-05

AF/1648
JFW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re United States Patent Application of:		**Docket No.:**	**4171-102 CIP**
Appellant:	**Jason C.H. SHIH**	**Examiner:**	**Zachariah LUCAS**
Serial No.:	**10/007,613**	**Art Group:**	**1648**
Date Filed:	**October 26, 2001**	**Confirm. No.:**	**4213**
Title:	**METHOD AND COMPOSITION FOR STERILIZING SURGICAL INSTRUMENTS**	**Customer No.:**	**23448**

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the Commissioner for Patents on the date specified, in an envelope addressed to Mail Stop Appeal Brief – Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, and Express Mailed under the provisions of 37 CFR 1.10.

Kate Turner

August 25, 2005
Date

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CORRECTED BRIEF ON APPEAL IN RESPONSE TO AUGUST 10, 2005 OFFICE COMMUNICATION IN U. S. PATENT APPLICATION NO. 10/007,613

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the August 10, 2005 Office Communication requesting a corrected Appeal Brief according to the rules set forth in 37 CFR 41.37, applicant submits three copies of the corrected Appeal Brief. Applicant has already submitted the required fee for entry of the Appeal Brief on

January 3, 2005 and as such no additional fee is due.

Respectfully submitted,

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BRIEF ON APPEAL

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal under 35 U.S.C. §134 from the Final Rejection in the Office Action dated June 30, 2004 Office Action, of claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 of U.S. Patent Application No. 10/007,613.

REAL PARTY IN INTEREST

The real party in interest in this appeal is BioResource International, Inc., the owner of the invention and patent rights of this application, by virtue of an Assignment of U.S. Patent Application No. 10/007,613 recorded in the assignment records of the U.S. Patent and Trademark Office on October 26, 2001 at reel 012366, frame 0566 (3 pages).

RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellant, the Appellant's legal representative, or assignee, which will directly effect or be directly affected by or have a bearing on the Board's decision in this appeal.

STATUS OF CLAIMS

A complete listing of claims 1-83 of the present application is attached in **Claim Appendix** hereof, among which claims 1-51, 53-56, 63, 68, 69, 71, 73, 74-80, and 82 are currently pending in the subject application.

Process claims 1-38 have been withdrawn from consideration in response to the restriction requirement imposed by the Examiner in the September 9, 2003 Office Action. Appellant intends to rejoin the withdrawn process claims 1-38 under MPEP §821.04 at a later time when the elected product claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 are determined to be allowable.

Claims 68, 69, and 75-79 have been withdrawn from consideration as being directed to non-elected species of proteolytic enzymes. Since claim 39 is a generic claim that covers both elected and non-elected enzyme species, claims 68, 69, and 75-79 will be entitled to consideration upon allowance of such generic claim 39.

Remaining claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 have been finally rejected under 35 U.S.C. §103(a) by the Examiner in the June 30, 2004 Office Action, and such rejected claims are the subject of this appeal.

Claims 39-51, 53-56, 63, 71, 73, 74, 80 and 82 are being appealed herein.

STATUS OF AMENDMENTS

In the August 30, 2004 Response to Office Action, Appellant cancelled claims 52, 57-61, and 64-65, and amended claims 39, 51, 53-56, 71, 80, and 82.

Such cancellation and amendments of claims were subsequently approved by the Examiner and entered for purpose of appeal in the September 22, 2004 Advisory Action.[1]

With the timely submission of a Notice of Appeal and this Appeal Brief with the required fees, the Response filed August 30, 2004 has perfected the record of the instant application on appeal, and the rejected claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 as amended in such Response are the subject of this appeal to the Board.

SUMMARY OF THE INVENTION

The claimed invention of the present application broadly relates to systems and methods for disinfecting and sterilizing medical devices and like articles that are susceptible to contamination by infectious prion proteins, by **combining thermal treatment and enzymatic degradation**.

Specifically, the treated articles are heated to an elevated temperature and exposed to a proteolytic enzyme, either successively at two different durations or simultaneously.

The thermal treatment functions to render the infective prion protein proteolytically susceptible. The temperature for conducting such thermal treatment is below the pyrolytic destruction temperature of the infective prion protein, and preferably at least 40°C but not more than 150°C.

The enzymatic degradation uses a thermally stable proteolytic enzyme, such as keratinase or subtilisin, for reducing or degrading the infective prion protein, which has been rendered proteolytically susceptible by the thermal treatment. The temperature for conducting such enzymatic degradation is preferably from about 50°C to about 65°C.

---

[1] The September 22, 2004 Advisory Action contains several self-evident typographic error: (a) Claim(s) rejected should be 39-51, 53-56, 63, 71, 73, 74, 80 and 82, instead of "35-51, 53-56, 63, 71, 73, 74, 80 and 82" as listed in Section (7) of the September 22, 2004 Advisory Action; (b) Claim(s) withdrawn from consideration should be 1-38, 68, 69 and 75-79, instead of "68, 69 and 75-79" as listed in Section (7) of the September 22, 2004 Advisory Action.

The specific features of the pending claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 are set out below in tabular format, cross-referenced to disclosure in the specification by page and line number, to facilitate the Board's review:

Claim No.	Features Recited by the Claim	Cross-Reference to the Specification
Claim 39	"A system comprising: (a) one or more articles susceptible to contamination by infectious prion protein; (b) means for heating said articles; (c) a proteolytic enzyme selected from the group consisting of keratinases and subtilisins; and (d) means for exposing said articles to said proteolytic enzyme, wherein said one or more articles are characterized by a first elevated temperature of at least 40°C and not more than about 150°C during a first duration, wherein said articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to said proteolytic enzyme during a second, subsequent duration."	Page 4, lines 15-21; page 5, lines 1-19. Page 6, lines 8-21. Page 10, line 21; page 11, lines1-2. Page 6, lines 3 and 6.
Claim 40	"wherein the proteolytic enzyme comprises keratinase."	Page 6, line 14.
Claim 41	"wherein the keratinase is provided in a solution at a concentration within a range of from about 0.2 g/L to about 1.0 g/L."	Page 7, lines 3-5.
Claim 42	"wherein the solution comprises a solvent selected from the group consisting of distilled water, alcohol, buffer solution, and detergent solution."	Page 8, lines 11-13.
Claim 43	"wherein said solution further comprises one or more chemical additives selected from the group consisting of surfactants, builders, boosters, and fillers."	Page 8, lines 14-17.
Claim 44	"wherein said articles comprise surgical instruments."	Page 5, lines 1-2.
Claim 45	"wherein said surgical instrument(s) are selected from the group consisting of: clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors, excavators, needle holders, suction tubes, trocars, coagulation electrodes, electroencephalographic depth electrodes, rib and	Page 5, lines 2-5.

Claim No.	Features Recited by the Claim	Cross-Reference to the Specification
	sternum spreaders, bipolar probes, and rib shears."	
Claim 46	"wherein said articles comprise cutleries and kitchen utensils."	Page 5, lines 7-8.
Claim 47	"wherein said cutleries and kitchen utensils are selected from the group consisting of: knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers."	Page 5, line 9.
Claim 48	"wherein said laboratory apparatuses are selected from the group consisting of: containers, filtration devices, centrifuges, spectrophotometers, and fluorometers."	Page 5, lines 14-19.
Claim 49	"wherein said article(s) comprise veterinary devices."	Page 5, line 12.
Claim 50	"wherein said veterinary devices are selected from the group consisting of clamps, forceps, knives, saws, probes, and electronic stun equipment."	Page 5, lines 12-13.
Claim 51	"wherein said first elevated temperature is higher than said second elevated temperature."	Page 6, lines 3-4.
Claim 53	"wherein said first elevated temperature is at least about 60°C."	Page 11, lines 1-2.
Claim 54	"wherein said first elevated temperature is in a range of from about 100°C to about 150°C."	Page 11, line 3.
Claim 55	"wherein said first elevated temperature is at least about 75°C."	Page 11, line 2.
Claim 56	"A system comprising: (a) one or more articles susceptible to contamination by infectious prion protein; (b) means for heating said one or more articles; (c) a proteolytic enzyme selected from the group consisting of keratinases and subtilisins; and (d) means for exposing said articles to said proteolytic enzyme; wherein said one or more articles are characterized by an elevated temperature of from about 40°C to about 60°C and exposure to said proteolytic enzyme."	Page 4, lines 15-21; page 5, lines 1-19; page 11, lines 5-16. Page 6, lines 8-21. Page 15, lines 16-17.
Claim 63	"wherein the proteolytic enzyme comprises a keratinase enzyme."	Page 6, line 14.

Claim No.	Features Recited by the Claim	Cross-Reference to the Specification
Claim 71	"A system comprising (a) a surgical instrument contaminated with infective prior protein; (b) means for heating the surgical instrument; (c) a proteolytic enzyme that is thermally stable at a temperature in a range of from about 35°C to about 100°C and proteolytically effective to at least partially destroy the infective prion protein contaminating said surgical instrument, and (d) means for exposing the surgical instrument to the proteolytic enzyme, wherein said surgical instrument is characterized by a first elevated temperature in a range of from about 100°C to about 150°C during a first duration, and wherein said surgical instrument is characterized by a second elevated temperature in a range of from about 35°C to about 100°C and exposure to said proteolytic enzyme during a second, subsequent duration."	Page 4, lines 15-21. Page 6, line 4; page 12, lines 10-12. Page 11, line 3. Page 6, line 4.
Claim 73	"wherein the proteolytic enzyme comprises at least one enzyme selected from the group consisting of keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin."	Page 12, lines 18-21; page 13, lines 1-2.
Claim 74	"wherein the proteolytic enzyme comprises *Bacillus licheniformis* PWD-1 keratinase."	Page 13, lines 3-4.
Claim 80	"A system comprising: (a) one or more articles susceptible to contamination by infectious prion protein; (b) means for heating said articles; (c) *Bacillus licheniformis* PWD-1 keratinase; and (d) means for exposing the heated articles to the *Bacillus licheniformis* PWD-1 keratinase, wherein said articles are characterized by a first elevated temperature of at least 40°C and not more than about 150°C during a first duration, and wherein said articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to the *Bacillus licheniformis* PWD-1 keratinase during a second, subsequent duration."	Page 4, lines 15-21; page 5, lines 1-19. Page 13, lines 3-4. Page 10, line 21; page 11, lines1-2. Page 6, lines 3 and 6.
Claim 82	"A system comprising: (a) one or more articles susceptible to	Page 4, lines 15-21; page 5, lines 1-19; page 11, lines 5-16

Claim No.	Features Recited by the Claim	Cross-Reference to the Specification
	contamination by infectious prion protein; (b) means for heating said articles; (c) *Bacillus licheniformis* PWD-1 keratinase; and (d) means for exposing the articles to *Bacillus licheniformis* PWD-1 keratinase, wherein said articles are characterized by an elevated temperature of from about 40°C to about 60°C and exposure to the *Bacillus licheniformis* PWD-1 keratinase."	Page 13, lines 3-4. Page 15, lines 16-17.

REFERENCES

The following references (copies of publications, not US Patents, in Evidence Appendix) were cited under 35 U.S.C. §103(a) in the June 30, 2004 Office Action finally rejecting the pending claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82:

(a) WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation, WORLD HEALTH ORGANIZATION (WHO), March 23-26, 1999 (hereinafter "WHO Document");

(b) **Huth** et al. U.S. Patent No. 6,448,062 (hereinafter "Huth");

(c) **Vlass** et al. U.S. Patent No. 6,210,639 (hereinafter "Vlass");

(d) **Potgeiter** et al. U.S. Statutory Invention Registration No. H1,818 (hereinafter "Potgeiter");

(e) **Shih** U.S. Patent No. 5,171,682 (hereinafter "Shih");

(f) **Bolton** et al., Molecular Characteristics of the Major Scrapie Prion Protein (hereinafter "Bolton"); and

(g) **Oesch** et al. Properties of the Scrapie Prion Protein: Quantitative Analysis of Protease Resistance (hereinafter "Oesch").

ISSUES

In the September 22, 2004 Advisory Action, the Examiner has withdrawn various previously-raised claim rejections, including the rejections for lack of enablement and new matter.

The only two remaining issues are:

(1) Whether it is appropriate for claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 to recite systems that include articles susceptible to contamination by infectious prion protein.

(2) Whether claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 are unpatentable under 35 U.S.C. §103(a) as being obvious over the WHO Document as the primary reference, in view of numerous secondary references including **Huth**, **Vlass**, **Potgeiter**, **Shih**, **Bolton**, and **Oesch**.

GROUPING OF THE CLAIMS

Group I: Claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 constitute a unitary group of claims presenting common issues in respect of their patentability. Claim 39 is representative of the group.

ARGUMENT

Issue 1 - Propriety of Claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 in Reciting Systems that Include Articles Susceptible to Contamination by Infectious Prion Protein – Claim 39 is Representative

In the June 30, 2004 Office Action, the Examiner objected to claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82, on the ground that such claims are directed to systems for treating articles that may be infected with prion proteins, and that it is inappropriate for such claims to include the articles to be treated in the claimed systems for treating the articles.

In response to such objection, the Appellant has amended claims 39 (from which claims 40-51, 53-55, and 63 depend), 56, 71 (from which claims 73 and 74 depend), 80 and 82 in the August 30, 2004 Response to recite simply **a system**, which **comprises** one or more articles susceptible to contamination by infectious prion protein, means for heating such articles, a proteolytic enzyme, and means for exposing such articles to the proteolytic enzyme (see claims 39, 56, 71, 80, and 82 as amended).

As a result of such amendments, claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 are no longer limited to systems or apparatuses for treating prion-contaminated articles. Instead, they are now directed to **a system that includes both the contaminated articles and means for treating such articles**, and therefore overcome the Examiner's objections.

The Examiner's assertion in the September 22, 2004 Advisory Action that such amended claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 still read on system for the treatment of articles that may be infected by prion proteins (see Advisory Action, page 2, second paragraph) is inconsistent with the claim language as amended in the August 30, 2004 Response and is incorrect.

Appellant therefore respectfully requests that the Board reverse the Examiner's objection to claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82.

Issue 2 - Patentability of Claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 under 35 U.S.C. §103(a) over the WHO Document in view of Huth, Vlass, Potgeiter, Shih, Bolton, and/or Oesch – Claim 39 is Representative

This rejection is traversed because the Examiner failed to establish a *prima facie* case of obviousness to support such rejection.

The Office has the initial burden of showing a *prima facie* case of obviousness. *In re Bell*, 26 U.S.P.Q.2d 1529, 1530 (Fed. Cir. 1993). In order to properly establish a *prima facie* case of obviousness based on combination of several references, the Examiner must show a reason, suggestion, or motivation to lead an inventor to combine those references. *Pro-Mold and Tool Co. V. Great Lakes Plastics Inc.*, 37 USPQ2d 1626, 1629 (Fed. Cir. 1996).

The representative claim 39 expressly requires:

> "A system comprising:
>
> (a) **one or more articles susceptible to contamination by infectious prion protein**;
>
> (b) **means for heating said articles**;
>
> (c) **a proteolytic enzyme selected from the group consisting of keratinases and subtilisins**; and
>
> (d) **means for exposing said articles to said proteolytic enzyme**,
>
> wherein said one or more articles are characterized by a first elevated temperature of at least 40°C and not more than about 150°C during a first duration, wherein **said articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to said proteolytic enzyme during a second, subsequent duration**."

The language of claim 39 expressly requires that the prion-contaminated articles be "characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to said proteolytic enzyme during a second, subsequent duration."

Such express requirement in claim 39 further imposes **an implicit structural limitation**. Such system must provide **a specific arrangement of the recited elements, i.e., the articles, the heating means, the proteolytic enzyme, and the exposing means, to enable simultaneous heating and enzymatic digestion** of the prion-contaminated articles, so that the articles can be characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to the proteolytic enzyme during a second, subsequent duration, as required by claim 39.

The cited references, either taken singularly or in combination, do not provide any derivative basis for such specific arrangement of articles, heating means, proteolytic enzyme, and exposing means, to allow the prion-contaminated articles to be characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to the proteolytic enzyme during a second, subsequent duration, as expressly required by claim 39.

The primary reference cited by the Examiner, i.e., the WHO document, discloses sterilization of prion-contaminated surgical instruments by boiling or autoclaving with sodium hydroxide or sodium hypochlorite, followed by subsequent routine sterilization (see page 29, Appendix III, section 2 of the WTO document).

The Examiner conceded that the WHO Document does not teach usage of proteolytic enzyme for treating prion-contaminated articles, but attempted to remedy such deficiency of the WHO Document by combining teachings by various secondary references including Huth, Vlass, Potgeiter, Shih, Bolton, and/or Oesch about the use of proteolytic enzyme.

However, such hypothetical combination proposed by the Examiner only yields **a system containing a mere aggregate of** articles, heating means, proteolytic enzyme and exposing means, but **it does not provide any derivative basis for a specific arrangement of such elements** that enables simultaneous heating and enzyme exposure of the prion-contaminated articles in such manner that such articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to the proteolytic enzyme during a second, subsequent duration, as expressly required by claim 39 of the present application. In fact, none of the secondary references acknowledges, or even

recognizes, the advantages of arranging the articles, the heating means, the proteolytic enzyme and the exposing means for simultaneous heating and enzyme exposure to allow the articles to be at an elevated temperature in a range of from about 50°C to about 65°C during exposure to a proteolytic enzyme.

In the September 22, 2004 Advisory Action, the Examiner asserted that claim 39 of the present application does not structurally distinguish over the cited prior art references, on the basis that there are no teachings demonstrating that the system suggested by the combination of the cited prior art references would not be capable of performing the functions of simultaneous heating and enzyme exposure.

However, it is clear that a specific arrangement of articles, heating elements, proteolytic enzyme, and exposing means is necessary for a system to perform the functions of simultaneous heating and exposing the articles to the proteolytic enzyme.

The system suggested by the combination of the cited prior art reference does not have such specific arrangement of articles, heating elements, proteolytic enzyme, and exposing means. Therefore, such prior art system is incapable of performing the functions of simultaneous heating and enzyme exposure.

Further, it has been well-established that when the claimed invention contains functional limitations not suggested by the prior art reference, **the mere fact that the prior art could be so modified to perform such functions would not have made the modification obvious, unless the prior art suggested the desirability of the modification**. See *In re Gordon*, 733 F.2d 900, 902, 221 UPSQ 1125, 1127 (Fed. Cir. 1984); see also *In re Mills*, 16 USPQ2d 1430 (CAFC 1990).

In this case, nothing in the cited references suggests the desirability of modifying the prior art system and re-arranging the prion-contaminated articles, the heating elements, the proteolytic enzyme, and the exposing means so as to allow simultaneous heating and enzyme exposure of the articles so that such articles are at elevated temperature in a range of from about 50°C to about 65°C during exposure to a proteolytic enzyme.

Therefore, claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 of the present application patentably distinguish over all cited references.

It therefore is respectfully requested that the Board take cognizance of the absence of any proper basis of the §103 rejection of claim 39, as representative of appealed claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82, and correspondingly reverse the Examiner's rejection of such claims.

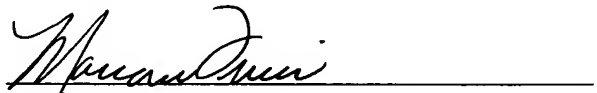
CONCLUSION

Based on the foregoing arguments and cited legal precedent, it is respectfully requested that the Board of Patent Appeals and Interferences reverse the decision of the Examiner finally rejecting claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 now pending in the application, consistent with the patentability of such claims over the cited art references.

This brief is provided in triplicate. No oral hearing is requested.

Enclosed with this appeal brief is a Credit Card Payment form, authorizing the Office to charge the office fee in the amount of $250.00 under 37 C.F.R. §1.17(c) to the credit card specified therein. Please charge any deficiency and credit any excess payment to Deposit Account No. 08-3284 of Intellectual Property/Technology Law.

Respectfully submitted,



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CLAIM APPENDIX

Appeal Claims 39-51, 53-56, 63, 71, 73, 74, 80, and 82 in a Listing of Claims 1-83

1. (Withdrawn) A method of disinfecting article(s) that are susceptible to contamination by infectious prion protein, the method comprising the steps of:

   (a) heating said article(s) to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein associated with said article(s); and

   (b) exposing the heated article(s) to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion associated with said article(s).

2. (Withdrawn) The method of claim 1, wherein said articles comprise surgical instruments.

3. (Withdrawn) The method of claim 2, wherein said surgical instrument(s) are selected from the group consisting of: clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors, excavators, needle holders, suction tubes, trocars, coagulation electrodes, electroencephalographic depth electrodes, rib and sternum spreaders, bipolar probes, and rib shears.

4. (Withdrawn) The method of claim 1, wherein said article(s) comprise cutleries and kitchen utensils.

5. (Withdrawn) The method of claim 4, wherein said cutleries and kitchen utensils are selected from the group consisting of: knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers.

6. (Withdrawn) The method of claim 1, wherein said article(s) comprise laboratory apparatus(es).

7. (Withdrawn) The method of claim 6, wherein said laboratory apparatus(es) are selected from the group consisting of: containers, filtration devices, centrifuges, spectrophotometers, and fluorometers.

8. (Withdrawn) The method of claim 1, wherein said article(s) comprise veterinary devices.

9. (Withdrawn) The method of claim 8, wherein said veterinary devices are selected from the group consisting of clamps, forceps, knives, saws, probes, and electronic stun equipment.

10. (Withdrawn) The method of claim 1, wherein the temperature in step (a) comprises a temperature not exceeding about 150°C.

11. (Withdrawn) The method of claim 1, wherein the temperature in step (a) comprises a temperature of at least 35°C.

12. (Withdrawn) The method of claim 1, wherein the temperature in step (a) comprises a temperature below about 150°C.

13. (Withdrawn) The method of claim 1, wherein the temperature in step (a) comprises a temperature in a range of from about 100°C to about 150°C.

14. (Withdrawn) The method of claim 1, wherein the temperature in step (a) comprises a temperature in a range of from about 125°C to about 140°C.

15. (Withdrawn) The method of claim 1, wherein step (b) is conducted at lower temperature than step (a).

16. (Withdrawn) The method of claim 1, wherein step (b) is carried out at temperature above about 40°C.

17. (Withdrawn) The method of claim 1, wherein step (b) is carried out at temperature above about 50°C.

18. (Withdrawn) The method of claim 1, wherein step (b) is carried out at temperature in a range of from about 35°C to about 75°C.

19. (Withdrawn) The method of claim 1, wherein step (b) is carried out at temperature in a range of from about 40°C to about 75°C.

20. (Withdrawn) The method of claim 1, wherein step (b) is carried out at temperature in a range of from about 50°C to about 65°C.

21. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises at least one enzyme selected from the group consisting of keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin.

22. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises a keratinase enzyme.

23. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises an active fragment of a keratinase enzyme.

24. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises a Bacillus licheniformis PWD-1 enzyme or an active fragment thereof.

25. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises a protease enzyme.

26. (Withdrawn) The method of claim 25, wherein the protease enzyme comprises a carbonyl hydrolase.

27. (Withdrawn) The method of claim 26, wherein the carbonyl hydrolase comprises subtilisin.

28. (Withdrawn) The method of claim 27, wherein the subtilisin comprises a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

29. (Withdrawn) The method of claim 25, wherein the protease enzyme comprises at least one enzyme selected from the group consisting of: papain, pancreatin, trypsin, chymotrypsin, pepsin, streptokinase, streptodornase, ficin, carboxypeptidase, aminopeptidase, chymopapain, bromelin, and subtilisin.

30. (Withdrawn) A method of removing infective prion protein from a surgical instrument contaminated with same, the method including (a) heating the surgical instrument at a temperature in a range of from about 100°C to about 150°C, followed by (b) exposing the heated surgical instrument to a proteolytic enzyme at a temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to at least partially destroy the infective prion protein contaminating said surgical instrument.

31. (Withdrawn) The method of claim 30, wherein said heating is conducted for a time of from about 5 minutes to about 5 hours.

32. (Withdrawn) The method of claim 30, wherein the proteolytic enzyme comprises at least one enzyme selected from the group consisting of keratinase enzymes, proteinase K, trypsins,

chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin.

33. (Withdrawn) The method of claim 30, wherein the proteolytic enzyme comprises *Bacillus licheniformis* PWD-1 keratinase.

34. (Withdrawn) The method of claim 1, wherein the proteolytic enzyme comprises a protease enzyme.

35. (Withdrawn) The method of claim 34, wherein the protease enzyme comprises a carbonyl hydrolase.

36. (Withdrawn) The method of claim 35, wherein the carbonyl hydrolase comprises subtilisin.

37. (Withdrawn) The method of claim 36 , wherein the subtilisin comprises a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

38. (Withdrawn) The method of claim 34, wherein the protease enzyme comprises at least one enzyme selected from the group consisting of: papain, pancreatin, trypsin, chymotrypsin, pepsin, streptokinase, streptodornase, ficin, carboxypeptidase, aminopeptidase, chymopapain, bromelin, and subtilisin.

39. (Previously presented) A system comprising:

(a) one or more articles susceptible to contamination by infectious prion protein;

(b) means for heating said articles;

(c) a proteolytic enzyme selected from the group consisting of keratinases and subtilisins; and

(d) means for exposing said articles to said proteolytic enzyme,

wherein said one or more articles are characterized by a first elevated temperature of at least 40°C and not more than about 150°C during a first duration, wherein said articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to said proteolytic enzyme during a second, subsequent duration.

40. (Previously presented) The system of claim 39, wherein the proteolytic enzyme comprises keratinase.

41. (Previously presented) The system of claim 40, wherein the keratinase is provided in a solution at a concentration within a range of from about 0.2 g/L to about 1.0 g/L.

42. (Previously presented) The system of claim 41, wherein the solution comprises a solvent selected from the group consisting of distilled water, alcohol, buffer solution, and detergent solution.

43. (Previously presented) The system of claim 42, wherein said solution further comprises one or more chemical additives selected from the group consisting of surfactants, builders, boosters, and fillers.

44. (Previously presented) The system of claim 39, wherein said articles comprise surgical instruments.

45. (Previously presented) The system of claim 44, wherein said surgical instrument(s) are selected from the group consisting of: clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors,

excavators, needle holders, suction tubes, trocars, coagulation electrodes, electroencephalographic depth electrodes, rib and sternum spreaders, bipolar probes, and rib shears.

46. (Previously presented) The system of claim 39, wherein said articles comprise cutleries and kitchen utensils.

47. (Previously presented) The system of claim 46, wherein said cutleries and kitchen utensils are selected from the group consisting of: knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers.

48. (Previously presented) The system of claim 47, wherein said laboratory apparatuses are selected from the group consisting of: containers, filtration devices, centrifuges, spectrophotometers, and fluorometers.

49. (Previously presented) The system of claim 39, wherein said article(s) comprise veterinary devices.

50. (Previously presented) The system of claim 49, wherein said veterinary devices are selected from the group consisting of clamps, forceps, knives, saws, probes, and electronic stun equipment.

51. (Previously presented) The system of claim 39, wherein said first elevated temperature is higher than said second elevated temperature.

52. (Cancelled).

53. (Previously presented) The system of claim 39, wherein said first elevated temperature is at least about 60°C.

54. (Previously presented) The system of claim 39, wherein said first elevated temperature is in a

range of from about 100°C to about 150°C.

55. (Previously presented) The system of claim 39, wherein said first elevated temperature is at least about 75°C.

56. (Previously presented) A system comprising:

(a) one or more articles susceptible to contamination by infectious prion protein;

(b) means for heating said one or more articles;

(c) a proteolytic enzyme selected from the group consisting of keratinases and subtilisins; and

(d) means for exposing said articles to said proteolytic enzyme;

wherein said one or more articles are characterized by an elevated temperature of from about 40°C to about 60°C and exposure to said proteolytic enzyme.

57-61. (Cancelled).

63. (Previously presented) The system of claim 39, wherein the proteolytic enzyme comprises a keratinase enzyme.

64-67. (Cancelled).

68. (Withdrawn) The system of claim 39, wherein the proteolytic enzyme comprises subtilisin.

69. (Withdrawn) The system of claim 68, wherein the subtilisin comprises a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

70. (Cancelled).

71. (Previously presented) A system comprising (a) a surgical instrument contaminated with infective prior protein; (b) means for heating the surgical instrument; (c) a proteolytic enzyme that is thermally stable at a temperature in a range of from about 35°C to about 100°C and proteolytically effective to at least partially destroy the infective prion protein contaminating said surgical instrument, and (d) means for exposing the surgical instrument to the proteolytic enzyme, wherein said surgical instrument is characterized by a first elevated temperature in a range of from about 100°C to about 150°C during a first duration, and wherein said surgical instrument is characterized by a second elevated temperature in a range of from about 35°C to about 100°C and exposure to said proteolytic enzyme during a second, subsequent duration.

72. (Cancelled).

73. (Previously presented) The system of claim 71, wherein the proteolytic enzyme comprises at least one enzyme selected from the group consisting of keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin.

74. (Previously presented) The system of claim 71, wherein the proteolytic enzyme comprises *Bacillus licheniformis* PWD-1 keratinase.

75. (Withdrawn) The system of claim 71, wherein the proteolytic enzyme comprises a protease enzyme.

76. (Withdrawn) The system of claim 75, wherein the protease enzyme comprises a carbonyl hydrolase.

77. (Withdrawn) The system of claim 76, wherein the carbonyl hydrolase comprises subtilisin.

78. (Withdrawn) The system of claim 77, wherein the subtilisin comprises a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

79. (Withdrawn) The system of claim 75, wherein the protease enzyme comprises at least one enzyme selected from the group consisting of: papain, pancreatin, trypsin, chymotrypsin, pepsin, streptokinase, streptodornase, ficin, carboxypeptidase, aminopeptidase, chymopapain, bromelin, and subtilisin.

80. (Previously presented) A system comprising:

(a) one or more articles susceptible to contamination by infectious prion protein;

(b) means for heating said articles;

(c) *Bacillus licheniformis* PWD-1 keratinase; and

(d) means for exposing the heated articles to the *Bacillus licheniformis* PWD-1 keratinase,

wherein said articles are characterized by a first elevated temperature of at least 40°C and not more than about 150°C during a first duration, and wherein said articles are characterized by a second elevated temperature in a range of from about 50°C to about 65°C and exposure to the *Bacillus licheniformis* PWD-1 keratinase during a second, subsequent duration.

81. (Cancelled).

82. (Previously presented) A system comprising:

(a) one or more articles susceptible to contamination by infectious prion protein;

(b) means for heating said articles;

(c) *Bacillus licheniformis* PWD-1 keratinase; and

(d) means for exposing the articles to *Bacillus licheniformis* PWD-1 keratinase,

wherein said articles are characterized by an elevated temperature of from about 40°C to about 60°C and exposure to the *Bacillus licheniformis* PWD-1 keratinase.

83. (Cancelled).

EVIDENCE APPENDIX

Statement of Evidence

Applicant submitted the enclosed Affidavit under 37 CFR §1.32 on April 16, 2004 and the Examiner entered this Affidavit by June 30, 2004.

Copies of the following references were relied on by Examiner and are included herewith:

(a) WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation, WORLD HEALTH ORGANIZATION (WHO), March 23-26, 1999 (hereinafter "WHO Document");

(f) **Bolton** et al., Molecular Characteristics of the Major Scrapie Prion Protein (hereinafter "Bolton"); and

(g) **Oesch** et al. Properties of the Scrapie Prion Protein: Quantitative Analysis of Protease Resistance (hereinafter "Oesch").

All U.S. Patents have not been included because of easy access to same in the USPTO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		**Docket No.:**	**4171-102 CIP**
Applicant:	**Jason C.H. Shih**	**Examiner:**	**Zachariah Lucas**
Application No.:	**10/007,613**	**Art Group:**	**1648**
Date Filed:	**October 26, 2001**	**Confirm. No.:**	**4213**
Title:	**METHOD AND COMPOSITION FOR STERILIZING SURGICAL INSTRUMENTS**	**Customer No.:**	**23448**

23448

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the Commissioner for Patents on the date specified, in an envelope addressed to Mail Stop Non-Fee Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, and Express Mailed under the provisions of 37 CFR 1.10.

Candace White

April 16, 2004
Date

EO 001 737 579 US
Express Mail Label Number

AFFIDAVIT UNDER 37 C.F.R. §1.132 OF DR. JASON C.H. SHIH IN U.S. PATENT APPLICATION NO. 10/007,613

Mail Stop Non-Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, **DR. JASON C.H. SHIH**, being duly sworn, depose and say:

(1) THAT I am the inventor and applicant for U.S. Patent Application No. 10/007,613, filed on October 26, 2001 in the U.S. Patent and Trademark Office in my name for "**METHOD AND COMPOSITION FOR STERILIZING SURGICAL INSTRUMENTS**" (hereinafter "the Application").

(2) THAT the Application discloses and claims a method and a system for disinfecting articles that are susceptible to contamination by infectious prion protein, wherein the articles to be disinfected is first heated to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infectious prion protein associated therewith, and then exposed to a proteolytic enzyme that is effective for at least partial reduction of the infectious prior protein (hereinafter "the Invention").

(3) THAT at my direction, experiments have been conducted to show the efficacy of various proteolytic enzymes in destructing or at least partially reducing infective prion protein in tissue samples at various concentrations with various pre-heating temperatures, according to the following experimental procedure:

> Eight (8) different solutions containing proteolytic enzymes at different concentrations were used to effect degradation and reduction of infectious prion protein responsible for Chronic Wasting Disease ("CWD") in deer and elk, as contained in infected deer brain tissues, after such infected tissues had been pre-heated to different temperatures. The Bio-Rad CWD Antigen Test Kit (Bio-Rad Laboratories at Hercules, CA) was used to determine the presence and concentration of the infectious prion protein in each sample after heat and enzyme treatment.

(4) THAT the above-mentioned eight proteolytic enzyme solutions included:

(a) a solution (A) containing no proteolytic enzyme, in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(b) a solution (B) containing purified PWD-1 keratinase ("KE") to yield a final concentration of about 50 mg/L in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(c) a solution (C) containing KE to yield a final concentration of about 100 mg/L in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(d) a solution (D) containing KE to yield a final concentration of about 200 mg/L in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(e) a solution (E) containing Subtilisin ("PS"), to yield a final concentration of 200 mg/L in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(f) a solution (F) containing crude PWD-1 keratinase ("CKE") to yield a final concentration of about 50 mg/L, together in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine;

(g) a solution (G) containing crude PWD-1 keratinase ("CKE") to yield a final concentration of about 100 mg/L, together in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine; and

(h) a solution (H) containing crude PWD-1 keratinase ("CKE") to yield a final concentration of about 200 mg/L, together in 50mM phosphate buffer, pH 8.0 and 2% N-lauroylsarcosine.

(5) THAT .350 g of CWD-positive deer brain stem tissue was mixed and homogenized in 1.5 mL of homogenization buffer, which was then separated into .500 mL aliquots and pre-heated for 40 minutes at a pre-heating temperature selected from 50°C, 80°C, 90°C, 100°C and 115°C. Samples subject to pre-heating at 115°C were pre-heated for 5 minutes only. Subsequently, the pre-heated tissue sample was

cooled and then treated with the respective proteolytic enzyme solution to yield the appropriate final concentration of enzyme. The pre-heating and enzyme treatment cycle was repeated for each of the eight different proteolytic enzyme solutions with each of the five different pre-heating temperatures. Enzymatic digestion was carried out according to the following:

(1) for KE: digestion at 50°C for 1 hour;

(2) for PS: digestion at 50°C for 1 hour; and

(3) for CKE: digestion at 50°C for 1 hour.

Each enzymatic digestion reaction was stopped by heating for 5 min at 80°C.

(6) THAT four (4) control samples were employed, which included:

(i) 500 µl BioRad Negative Control, (internal negative control provided in the testing kit);
(ii) 500 µl BioRad Positive Control, (internal positive control provided in the testing kit);
(iii) 500 µl Tissue Negative Control, (CWD-negative deer brain stem tissue); and
(iv) 500 µl Tissue Positive Control, (CWD-postive deer brain stem tissue).

(7) THAT all tissue samples and control samples were then examined by enzyme-linked immunosorbent assay (ELISA), according instructions provided in the Bio-Rad CWD Antigen Test Kit.

(8) THAT all the above-described tests, including both the sample tests and control tests, were carried out twice, and the results of which were correspondingly numbered as PLATE 1 and PLATE 2.

(9) THAT the ELISA test results were as follows:

PLATE 1

Lot No.	Protease	Conc.(mg/L)	Temperatures (°C)					Control	
			50	80	90	100	115	Negative	Positive
A			2.606	2.825	2.328	0.386	0.249	0.020	
B	KE	50	2.136	3.012	1.289	0.167	0.107	0.021	
C	KE	100	2.000	2.589	1.146	0.059	0.156	0.020	
D	KE	200	2.527	1.851	0.579	0.046	0.086	0.021	
E	PS	200	0.411	0.364	0.095	0.026	0.107		1.180
F	CKE	50	1.588	0.431	0.427	0.052	0.102		1.161
G	CKE	100	1.226	0.678	0.269	0.049	0.052	0.023	
H	CKE	200	0.987	0.802	0.111	0.060	0.043		3.340

PLATE 2

Lot No.	Protease	Conc.(mg/L)	Temperatures (°C)					Control	
			50	80	90	100	115	Negative	Positive
A			3.311	3.313	3.310	0.265	0.067	0.020	
B	KE	50	3.162	2.599	2.507	0.224	0.032	0.024	
C	KE	100	2.348	1.733	1.169	0.170	0.058	0.021	
D	KE	200	1.881	1.512	0.585	0.060	0.119	0.022	
E	PS	200	0.558	0.261	0.149	0.035	0.065		1.185
F	CKE	50	1.072	0.994	0.331	0.051	0.066		1.175
G	CKE	100	0.810	0.547	0.355	0.042	0.028	0.023	
H	CKE	200	0.516	0.324	0.538	0.062	0.046		3.358

The Bio-Rad negative control test results are provided in rows A-D. The Bio-Rad positive control test results are provided in rows E-F. The tissue negative control test results are provided in row G, and the tissue positive control test results are provided in row H.

(11) THAT the above-tabulated test results evidenced destruction or at least reduction of infectious prion protein in deer brain stem tissue samples initially containing same (sample test results shown in the middle), when such samples were treated by preheating followed by enzymatic digestion, in accordance with the Invention, using enzyme species including keratinase and subtilisin at different pre-heating temperatures ranging from about 50°C to about 115°C.

(12) THAT the prion concentrations in the tissue samples were found to decrease in general with the increase in the pre-heating temperatures, when the same type of proteolytic enzyme species and the same enzyme concentration were used for enzymatic digestion. However, the decrease in prion concentrations leveled off when the pre-heating temperature increased from 100°C to 115°C, and in some cases the prion concentrations even increased, which indicates that pre-heating temperature achieves optimal results at about 100°C.

(13) THAT the prion concentrations in the tissue samples were found to decrease in general with the increase in the concentrations of enzyme solutions used for enzymatic digestion, when the same type of proteolytic enzyme species and the same pre-heating temperature were employed. At the same enzyme concentration, subtilisin and crude PWD-1 keratinase enzymes were both found to be more effective in reducing or destructing infectious CWD prion agent than the purified PWD-1 keratinase.

(14) THAT the 200 mg/L subtilisin solution was effective in destructing from about 83% (as in PLATE 2) to about 84% (as in PLATE 1) of the infectious prion protein contained in a tissue sample that was preheated at a temperature of about 50°C.

(15) THAT the 200 mg/L crude PWD-1 keratinase solution was effective in destructing from about 62% (as in PLATE 1) to about 84% (as in PLATE 2) of the infectious prion protein contained in a tissue sample that was preheated at a temperature of about 50°C.

(16) THAT the 200 mg/L purified PWD-1 keratinase solution was effective in destructing from about 75% (as in PLATE 1) to about 82% (as in PLATE 2) of the infectious prion protein contained in a tissue sample that was preheated at a temperature of about 90°C.

Dr. Jason C.H. Shih

4/16/2004
Date

Dr. Jason C.H. Shih appeared before me on this 16th day of April, 2004. He declared to me that he is the person described in this Affidavit, and he executed this Affidavit before me, and declared that his execution was completely voluntary.

State of North Carolina
County of Wake

NOTARY PUBLIC
My Comm. Exp.
(SEAL)

Paulette R Jaeger
Notary Public

My commission expires: 11/10/08

WHO/CDS/CSR/APH/2000.3

WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies

Report of a WHO consultation
Geneva, Switzerland, 23-26 March 1999

World Health Organization
Communicable Disease Surveillance and Control

Contents

Section 1. INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal degenerative brain diseases that occur in humans and certain animal species. They are characterized by microscopic vacuoles and the deposition of amyloid (prion) protein in the grey matter of the brain. All forms of TSE are experimentally transmissible.

The following guideline on the prevention of iatrogenic and nosocomial exposure to TSE agents was prepared following the WHO Consultation on Caring for Patients and Hospital Infection Control in Relation to Human Transmissible Spongiform Encephalopathies, held in Geneva from 24 to 26 March 1999. The meeting was chaired by Dr Paul Brown. Dr Martin Zeidler and Dr Maurizio Pocchiari kindly agreed to be Rapporteurs. The full list of participants is given in Annex I. Presentations made at the Consultation are listed in Annex II.

This document provides guidance upon which infection control practitioners, healthcare practitioners, medical officers of health, and those involved in the care of persons suffering from TSE can base their care and infection control practices, to prevent events which are either extremely rare (e.g. transmission of TSE through a surgical procedure) or hypothetical (e.g. transmission of TSE to a healthcare worker or family member). Throughout the document there is specific and assumed reference to country or region-specific guidelines for matters which lie within the legal jurisdiction of that country or region, i.e. International Air Transport Association (IATA) regulations for transportation of hazardous goods, or bio-safety containment levels for laboratories. Readers should be familiar with such requirements for their own country or region.

Issues on which the consultants could not agree, or where the consultants did not feel there was sufficient expertise to render an opinion, have been noted. The consultation recognized that its recommendations to ensure maximum safety to care-givers and the environment may under some circumstances be regarded as impractical. However, they urged personnel involved with TSE patients or tissues to endeavour to comply as far as possible. There is no reason for a patient with a TSE to be denied any procedure, as any associated risks should be reduced to negligible levels by following the recommendations in this document.

Section 2. GENERAL CONSIDERATIONS

2.1 Transmissible Spongiform Encephalopathies in humans and in animals

Human TSEs occur in sporadic, familial, and acquired forms. The most common form, sporadic Creutzfeldt-Jakob disease (CJD), has a worldwide death rate of about 1 case per million people each year, and typically affects people between 55 and 75 years of age. The disease usually begins with a progressive mental deterioration that soon becomes associated with progressive unsteadiness and clumsiness, visual deterioration, muscle twitching (myoclonus), a variety of other neurological symptoms and signs, and is often associated with a characteristic periodic electroencephalogram. The patient is usually mute and immobile in the terminal stages and in most cases, death occurs within a few months of onset of symptoms. TSEs are invariably fatal and there is no proven treatment or prophylaxis.

Table 1 Human TSEs

Human TSE	First Reported
Creutzfeldt-Jakob Disease (CJD):[1]	
Sporadic (85-90%)	1921
Familial (5-10%)	1924
Iatrogenic (<5%)	1974
Variant (vCJD)	1996
Gerstmann-Sträussler-Scheinker Syndrome (GSS)	1936
Kuru	1957
Fatal Insomnia	
Familial	1986
Sporadic	1999

Similar neurodegenerative diseases also occur naturally in some animal species (scrapie in sheep and goats, chronic wasting disease in deer and elk), or as a result of exposure of susceptible species to infected animal tissues (transmissible mink encephalopathy, bovine spongiform encephalopathy, and spongiform encephalopathy in domestic cats and a variety of captive zoo animals).

TSE agents exhibit an unusual resistance to conventional chemical and physical decontamination methods. They are not adequately inactivated by most common disinfectants, or by most tissue fixatives, and some infectivity may persist under standard hospital or healthcare facility autoclaving conditions (e.g. 121°C for 15 minutes). They are also extremely resistant to high doses of ionizing and ultra-violet irradiation and some residual activity has been shown to survive for long periods in the environment. The unconventional nature of these agents, together with the appearance in the United Kingdom, Republic of Ireland and France of a new variant of CJD (vCJD) since the mid-1990s, has stimulated interest in an updated guidance on safe practices for patient care and infection control.

2.2 Diagnosis of Human Transmissible Spongiform Encephalopathies

The February 1998 Report of a WHO Consultation the Global Surveillance, Diagnosis and Therapy of Human Transmissible Spongiform Encephalopathies[2,3] provides a guideline for diagnostic criteria of human TSEs. Readers should be aware of efforts to revise diagnostic criteria for CJD and vCJD due to the introduction of new diagnostic tests and intense surveillance efforts. Surveillance case definitions (which may not be the same as diagnostic criteria) for both forms of the disease may also be subject to change.

2.3 Iatrogenic transmission

TSEs are not known to spread by contact from person to person, but transmission can occur during invasive medical interventions. Exposure to infectious material through the use of human cadaveric-derived pituitary hormones, dural and cornea homografts, and contaminated neurosurgical instruments has caused human TSEs. The Report of a

[1] Percentages vary somewhat from country to country.

[2] All cited WHO reports and consultations are available at the WHO Web site http://www.who.int/emc/diseases/bse/.

[3] WHO Consultation on Global Surveillance, Diagnosis and Therapy of Human Transmissible. Spongiform Encephalopathies. WHO/EMC/ZDI/98.9 Geneva, 9-11 February 1998.

WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies[4] can be consulted for more information and guidance on these issues.

2.4 Evaluating risk in healthcare environments

When considering measures to prevent the transmission of TSE from patients to other individuals (patients, healthcare workers, or other care providers), it is important to understand the basis for stipulating different categories of risk. Risk is dependent upon three considerations:

- the probability that an individual has or will develop TSE (see Section 2.4.1);
- the level of infectivity in tissues or fluids of these individuals (Section 2.4.2);
- the nature or route of the exposure to these tissues (Section 2.4.3).

From these considerations it is possible to make decisions about whether any special precautions are needed. Specific TSE decontamination procedures are described in Section 6. If TSE decontamination is required, the question remains as to how stringent it should be. The specific recommendations are described in sections devoted to Patient Care (Section 3), Occupational Injury (Section 4), Laboratory Investigations (Section 5) and Management After Death (Section 8).

2.4.1 Identification of persons for whom special precautions apply

Persons with confirmed or suspected TSEs are the highest risk patients. They must be managed using specific precautions which will be described in this and subsequent sections. All precautions recommended in the body of this document apply to the care of confirmed or suspect cases of TSE, or the handling of tissues from such patients, and unless otherwise noted, no distinction will be made between confirmed and suspect cases.

However, the concept of 'persons at risk for TSE' is useful in infection control, as it allows for the development of intermediate precautionary measures. The following persons have been regarded as 'at risk' for developing TSEs. The bracketed numbers are the number of reported occurrences of CJD transmitted through that route:

- recipients of dura mater (110 cases);
- recipients of human cadaver derived pituitary hormones, especially human cadaver derived growth hormone (130 cases);
- recipients of cornea transplants (3 cases - 1 definite, 1 probable, 1 possible);
- persons who have undergone neurosurgery (6);
- members of families with heritable TSE (5-10% of all cases of TSE are heritable, but the number of families varies widely from country to country).

The discussion and recommendations for healthy asymptomatic individuals considered to be at risk for TSE are described in Annex IV and referred to in Table 9.

The consultants did not extensively discuss the management of persons who have confirmed or suspected vCJD, due to the absence of specific data for review and the

[4] Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encepalopathies. Geneva, World Health Organization, 1997. WHO/EMC/ZOO/97.3 or WHO/BLG/97.2.

geographical isolation of the current cases. The discussion and their recommendations are described in Annex V and Table 9.

2.4.2 Tissue infectivity

From published and unpublished information, infectivity is found most often and in highest concentration in the central nervous system (CNS), specifically the brain, spinal cord and eye. This document will refer to these tissues as 'high infectivity tissues'.

Infectivity is found less often in the cerebrospinal fluid (CSF) and several organs outside the CNS (lung, liver, kidney, spleen/lymph nodes, and placenta). This document will refer to these tissues as 'low infectivity tissues'.

No infectivity has been detected in a wide variety of other tested tissues (heart, skeletal muscle, peripheral nerve, adipose tissue, gingival tissue, intestine, adrenal gland, thyroid, prostate, testis) or in bodily secretions or excretions (urine, faeces, saliva, mucous, semen, milk, tears, sweat, serous exudate). Experimental results investigating the infectivity of blood have been conflicting, however even when infectivity has been detectable, it is present in very low amounts and there are no known transfusion transmissions of CJD. This document will classify these tissues as having no detectable infectivity ('no detectable infectivity tissues') and, for the purposes of infection control, they will be regarded as non-infectious.

Table 2 Distribution of infectivity in the human body[5]

Infectivity Category	Tissues, Secretions, and Excretions	
High Infectivity	Brain Spinal cord Eye	
Low Infectivity	CSF Kidney Liver Lung Lymph nodes/spleen Placenta	
No Detectable Infectivity	Adipose tissue Adrenal gland Gingival tissue Heart muscle Intestine Peripheral nerve Prostate Skeletal muscle Testis Thyroid gland Blood[6]	Tears Nasal mucous Saliva Sweat Serous exudate Milk Semen Urine Faeces

[5] Assignment of different organs and tissues to categories of *high* and *low infectivity* is chiefly based upon the frequency with which infectivity has been detectable, rather than upon quantitative assays of the level of infectivity, for which data are incomplete. Experimental data include primates inoculated with tissues from human cases of CJD, but have been supplemented in some categories by data obtained from naturally occurring animal TSEs. Actual infectivity titres in the various human tissues other than the brain are extremely limited, but data from experimentally-infected animals generally corroborate the grouping shown in the table.

[6] See discussion this Section and Section 5.2.

The consultants agreed that an international effort to identify stored tissues from persons who later developed CJD or that were collected during the investigation for CJD (sporadic, iatrogenic or familial) should be initiated. These specimens should be tested in order to clarify the extent and level of infectivity during the pre-clinical phase of disease. Collections of these tissues, which are potentially infective, should be properly labelled as to their source and potential infectivity and appropriately stored to avoid cross contamination.

2.4.3 Route of exposure

When determining risk, infectivity of a tissue must be considered together with the route of exposure. Cutaneous exposure of intact skin or mucous membranes (except those of the eye) poses negligible risk; however, it is prudent and highly recommended to avoid such exposure when working with any high infectivity tissue. Transcutaneous exposures, including contact exposures to non-intact skin or mucous membranes,[7] splashes to the eye,[8] and inoculations via needle[9,10] or scalpel and other surgical instruments[11] pose a greater potential risk. Thus, it is prudent to avoid these types of exposures when working with either low infectivity or high infectivity tissues. CNS exposures (i.e. inoculation of the eye or CNS) with any infectious material poses a very serious risk, and appropriate precautions must always be taken to avoid these kinds of exposures.

Section 3. PATIENT CARE

3.1 Care of patients in the home and healthcare settings

3.1.1 Patient care

Normal social and clinical contact, and non-invasive clinical investigations (e.g. x-ray imaging procedures) with TSE patients do not present a risk to healthcare workers, relatives, or the community. There is no reason to defer, deny, or in any way discourage the admission of a person with a TSE into any healthcare setting. Based on current knowledge, isolation of patients is not necessary; they can be nursed in the open ward using Standard Precautions.

As the disease is usually rapidly progressive, the patient will develop high dependency needs and require ongoing assessment. It is essential to address the physical, nutritional, psychological, educational, and social needs of the patient and the associated needs of his or her family. Co-ordinated planning is vital in transferring care from one environment to another.

Private room nursing care is not required for infection control, but may be appropriate for compassionate reasons. Patient waste should be handled according to country, regional or federal regulations. Contamination by body fluids (categorized as no detectable infectivity tissues) poses no greater hazard than for any other patient. No special precautions are required for feeding utensils, feeding tubes, suction tubes, bed

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[7] TSE can be experimentally transmitted to healthy animals by exposing abraded gingival tissue to infected brain homogenate.

[8] By analogy with cornea transplants.

[9] A documented route of transmission in humans, from contaminated human cadaver extracted pituitary hormones (hGH and gonadotropin).

[10] Intraperitoneal, intramuscular and intravenous administration of *low infectivity tissue* extracts can cause transmission of TSE in experimental animals.

[11] By analogy with transmissions following neurosurgical procedures.

linens, or items used in skin or bed sore care in the home environment. Section 7 provides detailed information on disposal of medical waste.

3.1.2 Psychiatric manifestations

Caregivers both in the home and healthcare setting should be made aware and anticipate the possibility of labile psychiatric symptoms e.g. mood swings, hallucinations, or aggressive behavior. For this reason, training and counselling of professional and non-professional caregivers is recommended.

3.1.3 Confidentiality

Current heightened awareness requires special sensitivity to confidentiality of written and verbal communications. Special measures to safeguard the privacy of the patient and family are essential.

3.2 Dental procedures

Although epidemiological investigation has not revealed any evidence that dental procedures lead to increased risk of iatrogenic transmission of TSEs among humans, experimental studies have demonstrated that animals infected by intraperitoneal inoculation develop a significant level of infectivity in gingival and dental pulp tissues, and that TSEs can be transmitted to healthy animals by exposing root canals and gingival abrasions to infectious brain homogenate. The consultants agreed that the general infection control practices recommended by national dental associations are sufficient when treating TSE patients during procedures not involving neurovascular tissue. The committee was unable to come to a consensus on the risk of transmission of TSEs through major dental procedures; therefore, extra precautions such as those listed in Table 3 have been provided for consideration without recommendation.

Table 3 Optional precautions for major dental work

1.	Use single-use items and equipment e.g. needles and anaesthetic cartridges.
2.	Re-usable dental broaches and burrs that may have become contaminated with neurovascular tissue should either be destroyed after use (by incineration) or alternatively decontaminated by a method listed in Section 6 (Annex and III).
3.	Schedule procedures involving neurovascular tissue at end of day to permit more extensive cleaning and decontamination.

3.3 Diagnostic procedures

During the earlier stages of disease, patients with TSE who develop intercurrent illnesses may need to undergo the same kinds of diagnostic procedures as any other hospitalized patient. These could include ophthalmoscopic examinations, various types of endoscopy, vascular or urinary catheterization, and cardiac or pulmonary function tests. In general, these procedures may be conducted without any special precautions, as most tissues with which the instruments come in contact contain no detectable infectivity (see sub-Section 2.4.2). A conservative approach would nevertheless try to schedule such patients at the end of the day to allow more strict environmental decontamination (see Section 6.3) and instrument cleaning (see Section 6.2). When there is known exposure to high or low infectivity tissues, the instruments should be subjected to the strictest form of decontamination procedure which can be tolerated by the instrument. Instrument decontamination is discussed in more detail in Section 6.2 and decontamination methods are specifically described in Annex III.

3.4 Surgical procedures

Before admission to a hospital or healthcare facility, the infection control team should be informed of the intention to perform a surgical procedure on any person with confirmed or suspected TSE. Every effort should be made to plan carefully not only the procedure, but also the practicalities surrounding the procedure, e.g. instrument handling, storage, cleaning and decontamination or disposal. Written protocols are essential. All staff directly involved in these procedures or in the subsequent re-processing or disposal of potentially contaminated items, should be aware of the recommended precautions, and be adequately trained. The staff should be made aware of any such procedures in sufficient time to allow them to plan and to obtain suitable instruments and equipment (such as single use items), and it may be useful to schedule the patient at the end of the day's operating list. Staff must adhere to protocols that identify specifics regarding pre-operative, peri-operative and post-operative management of the patient, disposable materials, including bandages and sponges, and re-usable materials. Ancillary staff, such as laboratory and central instrument cleaning personnel, must be informed and appropriate training provided.

Basic protective measures are described in Table 4. Recommendations listed in Section 6 and Annex III for decontamination of equipment and environment, and in Section 7 for disposal of infectious waste should be followed. Supervisors should be responsible for ensuring that the appropriate procedures are followed and that effective management systems are in place.

Table 4 Precautions for surgical procedures

Wherever appropriate and possible, the intervention should:

1. be performed in an operating theatre;
2. involve the minimum required number of healthcare personnel;
3. use single-use equipment as follows:
   i) liquid repellent operating theatre gown, over a plastic apron
   ii) gloves
   iii) mask
   iv) visor or goggles
   v) linens and covers;
4. mask all non-disposable equipment;
5. maintain one-way flow of instruments;
6. treat all protective clothing, covers, liquid and solid waste by a method listed in Section 6; and Annex III; incineration is preferred
7. mark samples with a "Biohazard" label;
8. clean all surfaces according to recommendations specified in Section 6 and Annex III.

Procedures which are normally carried out at the bedside (e.g. lumbar puncture, bone marrow biopsy) may be performed at the bedside, but care should be taken to ensure ease of environmental decontamination should a spillage occur.

3.5 Handling of surgical instruments

3.5.1 General measures

Methods for instrument decontamination are fully discussed in Section 6. Determination of which method to use is based upon the infectivity level of the tissue and the way in which instruments will subsequently be re-used. For example, where surgical instruments contact high infectivity tissues, single-use surgical instruments are strongly recommended. If single-use instruments are not available, maximum safety is attained by destruction of re-usable instruments. Where destruction is not practical, re-usable instruments must be handled as per Table 5 and must be decontaminated as per Section 6 and Annex III.

Although CSF is classified as a low infectivity tissue and is less infectious than high infectivity tissues it was felt that instruments contaminated by CSF should be handled in the same manner as those contacting high infectivity tissues. This exception reflects the higher risk of transmission to any person on whom the instruments would be re-used for the procedure of lumbar puncture.

Table 5 General measures for cleaning instruments and environment

1.	Instruments should be kept moist until cleaned and decontaminated.
2.	Instruments should be cleaned as soon as possible after use to minimize drying of tissues, blood and body fluids onto the item.
3.	Avoid mixing instruments used on no detectable infectivity tissues with those used on high and low infectivity tissues.
4.	Recycle durable items for re-use only after TSE decontamination by methods found in Section 6 and Annex III.
5.	Instruments to be cleaned in automated mechanical processors must be decontaminated by methods described in Section 6 and Annex III before processing through these machines, and the washers (or other equipment) should be run through an empty cycle before any further routine use.
6.	Cover work surfaces with disposable material, which can then be removed and incinerated; otherwise clean and decontaminate underlying surfaces thoroughly using recommended decontamination procedures in Section 6 and Annex III.
7.	Be familiar with and observe safety guidelines when working with hazardous chemicals such as sodium hydroxide (NaOH, 'soda lye') and sodium hypochlorite (NaOCl, 'bleach') (see Annex III for definitions).
8.	Observe manufacturers' recommendations regarding care and maintenance of equipment.

Those instruments used for invasive procedures on TSE patients (i.e. used on high or low infectivity tissues) should be securely contained in a robust, leak-proof container labelled "Biohazard". They should be transferred to the sterilization department as soon as possible after use, and treated by a method listed in Annex III, or transferred to the incinerator as per Section 3.5.2. A designated person who is familiar with this guideline should be responsible for the transfer and subsequent management.

The consultation did not address the issue of post-exposure notification in the event that an instrument used on a high-risk tissue and/or high-risk patient was subsequently re-used without adequate decontamination.

3.5.2 Destruction of surgical instruments

Items for disposal by incineration should be isolated in a rigid clinical waste container, labelled 'Hazardous' and transported to the incinerator as soon as practicable, in line with the current disposal of clinical waste guidance described in the Teacher's Guide: Management of Wastes from Health-care Facilities[12] published by WHO. To avoid unnecessary destruction of instruments, quarantine of instruments while determining the final diagnosis of persons suspected of TSEs may be used.

3.5.3 Quarantine

If a facility can safely quarantine instruments until a diagnosis is confirmed, quarantine can be used to avoid needless destruction of instruments when suspect cases are later found not to have a TSE. Items for quarantine should be cleaned by the best non-destructive method as per Section 6 and Annex III, sterilized, packed, date and 'Hazard' labelled, and stored in specially marked rigid sealed containers.[13] Monitoring and ensuring maintenance of quarantine is essential to avoid accidental re-introduction of these instruments into the circulating instrument pool. If TSE is excluded as a diagnosis, the instruments may be returned to circulation after appropriate sterilization.

3.6 Anaesthesia

3.6.1 General anaesthesia

TSEs are not transmissible by the respiratory route; however, it is prudent to treat any instruments in direct contact with mouth, pharynx, tonsils and respiratory tract by a method described in Annex III. Destruction by incineration of non re-usable equipment is recommended.

3.6.2 Local anaesthesia

Needles should not be re-used, and in particular, needles contacting the CSF (e.g. for saddle blocks and other segmental anaesthetic procedures) must be discarded and destroyed.

3.7 Pregnancy and childbirth

TSE is not known to be transmitted from mother to child during pregnancy or childbirth; familial disease is inherited as a result of genetic mutations. In the event that a person with TSE becomes pregnant, no particular precautions need to be taken during the pregnancy, except during invasive procedures as per Section 3.4. Childbirth should be managed using standard infection control procedures, except that precautions should be taken to reduce the risk of exposure to placenta and any associated material and fluids. These should be disposed of by incineration. Instruments should be handled as for any other clinical procedure (Table 5). In home deliveries, the midwife (or any other persons in charge of delivery) should ensure that any contaminated material is removed and disposed of in accordance with correct procedures for infected clinical waste.

[12] Pruess A, Townend WK. Teacher's Guide: Management of Wastes from Health-care Activities. Geneva, World Health Organization, 1998. WHO/EOS/98.6.

[13] Although the intention of quarantine is to avoid destruction of instruments and will permit the re-introduction of instruments only if TSEs are not diagnosed, the use of a decontamination method for TSEs will confer additional safety should an instrument unintentionally come in contact with staff or patients.

Section 4. OCCUPATIONAL INJURY

4.1 Occupational exposure

Although there have been no confirmed cases of occupational transmission of TSE to humans, cases of CJD in healthcare workers have been reported in which a link to occupational exposure is suggested. Therefore, it is prudent to take a precautionary approach. In the context of occupational exposure, the highest potential risk is from exposure to high infectivity tissues through needle-stick injuries with inoculation; however exposure to either high or low infectivity tissues through direct inoculation (e.g. needle-sticks, puncture wounds, 'sharps' injuries, or contamination of broken skin) must be avoided. Exposure by splashing of the mucous membranes (notably the conjunctiva) or unintentional ingestion may be considered a hypothetical risk and must also be avoided. Healthcare personnel who work with patients with confirmed or suspected TSEs, or with their high or low infectivity tissues, should be appropriately informed about the nature of the hazard, relevant safety procedures, and the high level of safety which will be provided by the proposed procedures described throughout this document.

4.2 Post-exposure management

Appropriate counselling should include the fact that no case of human TSE is known to have occurred through occupational accident or injury. A number of strategies to minimize the theoretical risk of infection following accidents have been proposed, but their usefulness is untested and unknown. For the present the following common-sense actions are recommended:

- Contamination of unbroken skin with internal body fluids or tissues: wash with detergent and abundant quantities of warm water (avoid scrubbing), rinse, and dry. Brief exposure (1 minute, to 0.1N NaOH or a 1: 10 dilution of bleach) can be considered for maximum safety.
- Needle sticks or lacerations: gently encourage bleeding; wash (avoid scrubbing) with warm soapy water, rinse, dry and cover with a waterproof dressing. Further treatment (e.g., sutures) should be appropriate to the type of injury. Report the injury according to normal procedures for your hospital or healthcare facility/laboratory.
- Splashes into the eye or mouth: irrigate with either saline (eye) or tap water (mouth); report according to normal procedures for your hospital or healthcare facility/laboratory.
- Health and safety guidelines mandate reporting of injuries, and records should be kept for no less than 20 years.

Section 5. LABORATORY INVESTIGATIONS

5.1 Safety in the healthcare laboratory

Adherence to the following routine precautions during any diagnostic procedure or laboratory work will reduce the risk of infection. General protective measures and basic precautions as outlined in Table 6 are recommended for hospital-based diagnostic laboratories as well as during decontamination procedures in those laboratories. Detailed descriptions of these general protective measures can be found in the WHO document:

Safety in Health-care Laboratories[14] from which Table 6 is adapted. Where local or national regulations and guidelines exist, these should also be consulted. Only persons who have been advised of the potential hazards and who meet specific entry requirements (i.e. training) should be allowed to enter the laboratory working areas, or to participate in the collection of high infectivity tissues from patients with confirmed or suspected TSEs.

Table 6 General protective measures

1.	Eating, drinking, smoking, storing food and applying cosmetics must not be permitted in the laboratory work areas.
2.	Laboratory coveralls, gowns or uniforms must be worn for work and removed before entering non-laboratory areas; consider the use of disposable gowns; non-disposable gowns must be decontaminated by appropriate methods (see Section 7 Waste Disposal and Annex III).
3.	Safety glasses, face shields (visors) or other protective devices must be worn when it is necessary to protect the eyes and face from splashes and particles.
4.	Gloves appropriate for the work must be worn for all procedures that may involve unintentional direct contact with infectious materials. Armoured gloves should be considered in post mortem examinations or in the collection of high infectivity tissues.
5.	All gowns, gloves, face-shields and similar re-usable or non re-usable items must be either cleaned using methods set out in Annex III, or destroyed as per Section 7.
6.	Wherever possible, avoid or minimize the use of sharps (needles, knives, scissors and laboratory glassware), and use single-use disposable items.
7.	All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets.
8.	Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day, using methods described in Section 6 and Annex III.
9.	All contaminated materials, specimens and cultures must be either incinerated, or decontaminated using methods described in Section 6 and Annex III and Section 7 before disposal.
10.	All spills or accidents that are overt or potential exposures to infectious materials must be reported immediately to the laboratory supervisor, and a written record retained.
11.	The laboratory supervisor should ensure that adequate training in laboratory safety is provided and that practices and procedures are understood and followed.

5.2 Clinical diagnostic laboratories

The vast majority of diagnostic examinations in clinical laboratories are performed on blood (e.g. complete blood counts) and serum (e.g. chemistries), usually with automated analyzing equipment. As discussed in Section 2.4.2, blood and its components, although found to contain very low levels of infectivity in experimental models of TSE, have never been identified to be responsible for any case of CJD in humans, despite numerous exhaustive searches. The consultation felt that this epidemiological evidence was more relevant and more persuasive than the experimental evidence, and strongly recommended that blood specimens from patients with CJD not be considered to be infectious, and that no special precautions were needed for its handling in clinical laboratories. Similarly, except for CSF, other body fluids, secretions and excretions contain no infectivity, and need no special handling (Section 2.4.2, Table 2).

[14] Safety in Health-care Laboratories. Second Edition. Geneva, World Health Organization, 1992. ISBN 92 4 154450 3. This edition is under revision.

CSF may be infectious and must be handled with care. It is recommended that analysis not be performed in automated equipment, and any materials coming in contact with the CSF must either be incinerated or decontaminated according to one of the methods listed in Section 6 and Annex III. There is no reason for a diagnostic test to be denied if these measures are observed.

5.3 Surgical pathology

Although brain biopsy tissue is (at least historically) the most likely tissue from a patient with a TSE to be examined in the surgical pathology laboratory, it may also occur that other tissues are sent to the laboratory for examination, when patients with TSE undergo surgical procedures of one sort or another for intercurrent problems during the course of their neurological illness. The tissue categories of high infectivity, low infectivity, and no detectable infectivity are listed and discussed in Section 2.4.2 and Table 2. Precautions to be taken when handling different tissue specimens are presented in Table 7. Since histopathological processing of brain tissue is most often conducted upon autopsy (WHO does not recommend brain biopsy for the diagnosis of CJD), detailed instructions for histopathological processing are described in Section 8.2 (Post Mortem Examination, sub-Section 8.2.2, Histopathological Examination).

Table 7 Precautions for working with high and low infectivity tissues from patients with known or suspected TSEs

1. Whenever possible and where available, specimens should be examined in a laboratory or centre accustomed to handling high and low infectivity tissues; in particular, high infectivity tissue specimens should be examined by experienced personnel in a TSE laboratory.
2. Samples should be labelled 'Biohazard'.
3. Single-use protective clothing is preferred as follows:
   - liquid repellent gowns over plastic apron;
   - gloves (cut-resistant gloves are preferred for brain cutting);
   - mask;
   - visor or goggles.
4. Use disposable equipment wherever possible.
5. All disposable instruments that have been in contact with high infectivity tissues should be clearly identified and disposed of by incineration.
6. Use disposable non-permeable material to prevent contamination of the work surface. This covering and all washings, waste material and protective clothing should be destroyed and disposed of by incineration.
7. Fixatives and waste fluids must be decontaminated by a decontamination method described in Section 6 and Annex III or adsorbed onto materials such as sawdust and disposed of by incineration as a hazardous material.
8. Laboratories handling large numbers of samples are advised to adopt more stringent measures because of the possibility of increased residual contamination, e.g. restricted access laboratory facilities, the use of 'dedicated' microtomes and processing labware, decontamination of all wastes before transport out of the facility for incineration.

Note: This document contains recommendations designed for healthcare laboratories and is not intended as a guideline for scientific research laboratories. WHO has identified a number of reference laboratories[15] which may be contacted for advice on safety protocols for investigational laboratory environments.

[15] Global Surveillance, Diagnosis and Therapy of Human Transmissible Spongiform Encephalopathies: Report of a WHO Consultation. Geneva, World Health Organization, 1998. WHO/EMC/ZDI/98.9.

5.4 Transport of specimens by air

The transportation of pathology samples by air must comply with the International Air Transport Association (IATA) Restricted Articles Regulations and any additional requirements of the individual carriers. Documentation required by the IATA includes Shipper's Certificate for Restricted Articles, which requires that the content, nature and quantity of infectious material to be disclosed. The WHO Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens[16] provides more information on the safe transport of material. Where properly packaged according to these guidelines, there is no danger to the carriers.

Section 6. DECONTAMINATION PROCEDURES

6.1 General considerations

TSE agents are unusually resistant to disinfection and sterilization by most of the physical and chemical methods in common use for decontamination of infectious pathogens. Table 8 lists a number of commonly used chemicals and processes that cannot be depended upon for decontamination, as they have been shown to be either ineffective or only partially effective in destroying TSE infectivity. Variability in the effectiveness appears to be highly influenced by the nature and physical state of the infected tissues. For example, infectivity is strongly stabilized by drying or fixation with alcohol, formalin or glutaraldehyde. As a consequence, contaminated materials should not be exposed to fixation reagents, and should be kept wet between the time of use and disinfection by immersion in chemical disinfectants.

Table 8 Ineffective or sub-optimal disinfectants

Chemical disinfectants	Gaseous disinfectants	Physical processes
Ineffective[17] alcohol ammonia ß-propiolactone formalin hydrochloric acid hydrogen peroxide peracetic acid phenolics sodium dodecyl sulfate (SDS) (5%)	Ineffective ethylene oxide formaldehyde	Ineffective boiling dry heat (<300°C) ionising, UV or microwave radiation
Variably or partially effective chlorine dioxide glutaraldehyde guanidinium thiocyanate (4 M) iodophores sodium dichloro-isocyanurate sodium metaperiodate urea (6 M)		Variably or partially effective autoclaving at 121°C for 15 minutes boiling in 3% sodium dodecyl sulfate (SDS)

[16] Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens. Geneva, World Health Organization, 1997. WHO/EMC/97.3.

[17] Some of these chemicals may have very small effects on TSE infectivity and are not adequate for disinfection.

6.2 Decontamination of instruments

Policy makers should be guided by the infectivity level of the tissue contaminating the instrument and by the expectations of how the instrument will be re-used, as per Section 2.4. In this way, the most stringent recommendations are applied to instruments contacting high infectivity tissues of a person with a known TSE, which will also subsequently be re-used in the CNS or spinal column. Policy makers are encouraged to adopt the highest decontamination methods feasible until studies are published which clarify the risk of re-using decontaminated instruments.

Annex III lists the decontamination methods recommended by the consultation in order of decreasing effectiveness. It was emphasized that the safest and most unambiguous method for ensuring that there is no risk of residual infectivity on surgical instruments is to discard and destroy them by incineration. While this strategy should be universally applied to those devices and materials that are designed to be disposable, it was also recognized that this may not be feasible for many devices and materials that were not designed for single use. For these situations, the methods recommended in Annex III appear to remove most and possibly all infectivity under the widest range of conditions.

Those surgical instruments that are going to be re-used may be mechanically cleaned in advance of subjecting them to decontamination. Mechanical cleaning will reduce the bio-load and protect the instrument from damage caused by adherent tissues. If instruments are cleaned before decontamination, the cleaning materials must be treated as infectious waste, and the cleaning station must be decontaminated by one of the methods listed in Annex III. The instruments are then treated by one of the decontamination methods recommended in Annex III before reintroduction into the general instrument sterilization processes. A minority opinion held that instruments should be decontaminated before mechanical cleaning, and then handled as per general instrument sterilization processes.

Annex III recommends that, where possible, two or more different methods of inactivation be combined in any sterilization procedure for these agents. Procedures that employ heat and NaOH (either consecutively or simultaneously) appear to be sterilizing under worst-case conditions (e.g., infected brain tissue partly dried on to surfaces). Moreover, hot alkaline hydrolysis reduces biological macromolecules to their constituent sub-units, thereby cleaning as well as inactivating.

The consultation recognized that complex and expensive instruments such as intracardiac monitoring devices, fiberoptic endoscopes, and microscopes cannot be decontaminated by the harsh procedures specified in Annex III. Instead, to the extent possible, such instruments should be protected from surface contamination by wrapping or bagging with disposable materials. Those parts of the device that come into contact with internal tissues of patients should be subjected to the most effective decontaminating procedure that can be tolerated by the instrument. All adherent material must be removed and, if at all possible, the exposed surfaces cleaned using a decontamination method recommended in Annex III. Some instruments can be partly disassembled (e.g. drills and drill bits). Removable parts that would not be damaged by autoclaving, NaOH, or bleach should be dismounted and treated with these agents. In all instances where unfamiliar decontamination methods are attempted, the manufacturer should be consulted. These cleaning procedures should be applied even if the instrument has been re-used before discovery of its potential contamination.

Contaminated instruments or other contaminated materials should not be cleaned in automated washers without first having been decontaminated using a method recommended in Annex III.

6.3 Decontamination of work surfaces

Because TSE infectivity persists for long periods on work surfaces, it is important to use disposable cover sheets whenever possible to avoid environmental contamination, even though transmission to humans has never been recognized to have occurred from environmental exposure. It is also important to mechanically clean and disinfect equipment and surfaces that are subject to potential contamination, to prevent environmental build-ups. Surfaces contaminated by TSE agents can be disinfected by flooding, for one hour, with NaOH or sodium hypochlorite, followed by water rinses (see Annex III for detailed instructions). Surfaces that cannot be treated in this manner should be thoroughly cleaned; consider use of a partially effective method as listed in Table 8. Cleaning materials treated as potentially contaminated (see Section 6.4).

6.4 Decontamination of wastes and waste-contaminated materials

Decontamination of waste liquid and solid residues should be conducted with the same care and precautions recommended for any other exposure to TSE agents. The work area should be selected for easy containment of contamination and for subsequent disinfection of exposed surfaces. All waste liquids and solids must be captured and treated as infectious waste.

Liquids used for cleaning should be decontaminated in situ by addition of NaOH or hypochlorite or any of the procedures listed in Annex III, and may then be disposed of as routine hospital waste. Absorbents, such as sawdust, may be used to stabilize liquids that will be transported to an incinerator; however, this should be added after decontamination.

Cleaning tools and methods should be selected to minimize dispersal of the contamination by splashing, splatters and aerosols. Great care is required in the use of brushes and scouring tools. Where possible, cleaning tools such as brushes, towelling and scouring pads, as well as tools used for disassembling contaminated apparatus, should either be disposable or selected for their ability to withstand the disinfection procedures listed in Annex III.

Upon completion of the cleaning procedure, all solid wastes including disposable cleaning materials should be collected and decontaminated. Incineration is highly recommended. The cleaning station should then itself be decontaminated using one of the methods in Annex III.

Automated cleaning equipment must not be used for any instrument or material that has not previously been thoroughly decontaminated following the recommendations in Section 6.2 and Annex III.

6.5 Personal protection during decontamination procedures

Persons involved in the disinfection and decontamination of instruments or surfaces exposed to the tissues of persons with TSE should wear single-use protective clothing, gloves, mask and visor or goggles, as noted in Section 5.1, Table 6. The recommendations found in Table 6 can be adapted to different situations. All individuals involved with disinfection and decontamination procedures should be familiar with these basic protective measures and precautions. Handling of contaminated instruments during transfers and cleaning should be kept to a minimum.

6.6 Decontamination risk categories

The recommended levels of decontamination are shown in Table 9 for different patient and tissue risk categories (including patients at risk of TSE, and patients with vCJD). The table reflects the consensus of the consultation, and should be used in conjunction with Section 2.4.2 (Table 2) which lists specific high and low infectivity tissues, and Annex III, which describes specific decontamination options.

Table 9 Decontamination levels for different risk categories

Patient category	Tissue category	Decontamination options
Confirmed or suspect cases of TSE	High infectivity	Annex III
	Low infectivity	Annex III (but note that CSF, and peripheral organs and tissues are regarded as less infectious than the CNS)
Persons with known prior exposure to human pituitary derived hormones, cornea or dura mater grafts	High infectivity	Annex III
	Low Infectivity	Routine cleaning and disinfection procedures
Members of families with heritable forms of TSE	High Infectivity	No consensus was reached. The majority felt that TSE decontamination method should be used, but a minority felt this was unwarranted.
	Low Infectivity	Routine cleaning and disinfection procedures
All of the above categories	No detectable Infectivity	Routine cleaning and disinfection procedures
Confirmed or suspect cases of vCJD	All tissue categories	Annex III

Section 7. WASTE DISPOSAL

Infectious healthcare waste is defined as the discarded materials that have been in contact with blood and its derivatives, or wastes from infection isolation wards. These include but are not limited to cultures, tissues, dressings, swabs or other items soaked with blood, syringe needles, scalpels, diapers, and blood bags. The term 'TSE infectious healthcare waste' applies to high and low infectivity tissues from persons with confirmed or suspected TSEs, or high infectivity tissue from persons with known prior exposure to cornea, dura matter or human growth hormone, and any disposable items that have come in contact with these tissues.

In the absence of a national standard, disposal of biological waste contaminated by a TSE is to be performed in accordance with the best practice that is most consistent with this document or equivalent standards. Practitioners should review guidelines prescribed under the laws, procedures, codes of practice or other regulatory provisions in force in the relevant state or territory. All material classified as clinical waste should be placed in secure leak-proof containers and disposed of by incineration at an authorized incineration site. Avoid external contamination of the container to ensure safe handling of clinical

waste. The WHO guide, Safe Management of Wastes from Health Care Activities,[18] provides recommendations on medical and laboratory waste disposal.

TSE infectious waste should be incinerated or treated by a method that is effective for the inactivation of TSE agents (see Annex III). In regions where no incineration facilities are available, it is recommended that these wastes be chemically disinfected and then burnt in pits dedicated to final disposal. Residues should be checked for total combustion. Authorities should ensure that waste is adequately managed, as in certain big cities of the developing world it has been estimated that as much as one half of infectious waste is cleaned, re-packaged and sold in the marketplace.

In hospital or healthcare facility environments, drainage equipment, linens or swabs contaminated by high infectivity tissues or CSF should be collected into tough plastic bags or containers labelled 'Biohazard' and incinerated. Low infectivity tissues and drainage from low infectivity tissues[19] should be handled cautiously.

For tissues, secretions, or excretions with no detectable infectivity, no special requirements beyond Standard Precautions are required for the handling of body fluids or body-fluid contaminated linen, equipment or environments. Other infectious wastes from home care require no special precautions beyond those taken for any other disease. Sharp waste items (i.e. syringe needles) used during home care of TSE patients should be collected in impermeable containers and returned to the treating physician or healthcare establishment for disposal.

The use of enamel, heat-stable plastic or disposable trays when working with infectious specimens will help to confine contamination. If re-usable, they should be treated by a method listed in Annex III. Disposable items should be incinerated after use, although methods listed in Annex III may be used before disposal. Use absorbent material to soak up spills, which can then be contained and incinerated or treated by a method described in Annex III. Spills of potentially TSE infectious materials in the ward should be removed using absorbent material and the surface disinfected according to Annex III.

Use secure leak-proof containers, e.g. double bagging, for the safe handling of clinical waste. Avoid external contamination of the waste container. Disposable gloves and an apron should be worn when removing such spills and should subsequently be disposed of by incineration, together with the recovered waste and cleaning materials, although a method described in Annex III may be used.

Section 8. AFTER DEATH

8.1 Precautions for handling of the deceased patient

On the death of a patient with confirmed or suspected TSE, the removal of the body from the ward, community setting, or hospice, should be carried out using normal infection control measures. It is recommended that the deceased patient be placed in a sealed body bag prior to moving, in line with normal procedures for bodies where there is a known infection risk. Where the skull is open or there is CSF leakage, and where sutures do not completely control this leaking, the bag should be lined with materials to absorb any fluid, and the body should be moved in a sealed body bag. Refer to

[18] A. Prüss, E. Giroult, P. Rushbrook, eds. Safe Management of Wastes from Health Care Activies. Geneva, World Health Organization, 1999.

[19] Drainage from *low infectivity tissue* that has not been specifically tested for infectivity, however, may retain infectivity.

country-based guidelines and regulations for more information on care and handling of a deceased and infected patient.

8.2 Post mortem examination

Post mortem examinations remain an essential element in confirming the clinical diagnosis and the cause of death as TSE. Ideally, three people should be present during the examination: the pathologist assisted by one technician, and one further person to handle and label specimen containers. Except for training purposes, observers should be prohibited or kept to a minimum. All personnel should be made aware of the relevant history of the patient and fully informed of procedures for such post mortem examinations.

8.2.1 Conducting the autopsy

To the extent possible, disposable protective clothing should be worn including surgical cap and gown, apron, double gloves, and a face visor which completely encloses the operator's head to protect the eyes, nose and mouth. Consideration should be given to the use of hand protection, such as armoured or cut-resistant gloves.

Disposable or dedicated reuseable instruments are recommended in order to minimize the risk of environmental contamination. Manual saws are recommended in order to avoid the creation of tissue particulates and aerosols and for ease of decontamination after use. Electric saws, if used, should be operated inside an aerosol-containing bag unless ventilated helmets with an appropriate filter are worn. Instruments and mortuary working surfaces should be decontaminated following the guidance in Section 6 and Annex III.

Restricted post mortem examinations on TSE cases can be undertaken in any mortuary. If examination is limited to the brain, a plastic sheet with absorbent wadding and raised edges is first placed underneath the head to ensure containment of tissue debris and body fluids (e.g., CSF). The scalp is reflected in the normal way and the cranium is opened. After removal of the brain, replacement of the skullcap and suturing of the skin, the plastic sheet containing all tissue debris and drainage is bagged and sealed and sent for incineration. A full post mortem examination is discouraged except in dedicated facilities, unless special circumstances warrant the added difficulty of infectivity containment.

8.2.2 Histopathological examination

Only persons who have been advised of the potential hazards and trained in the specific methods used for TSE infectious tissues should be permitted to work in laboratories where high infectivity tissues are being processed. Facilities conducting a large number of histological examinations on high infectivity tissues should dedicate laboratory space, processors, instruments, glassware and reagents for this purpose. Guidelines in some countries and regions require Bio-Safety Containment Level 3 for handling these tissues.

It is important to note that formalin and glutaraldehyde-fixed TSE tissue retains infectivity for long periods, if not indefinitely. As a result, they should be handled with the same precautions as fresh material and be considered infectious throughout the entire procedure of fixation, embedding, sectioning, staining, and mounting on slides, until or unless treated with formic acid. Treatment with formic acid reduces infectivity to negligible levels. Although exact procedures may vary, formic acid treatment consists of

placing small pieces of fixed tissue, no more than 4 to 5 mm thick, in 50 to 100 ml of 95% formic acid for an hour, and then transferring them to fresh formalin for another two days before further processing. The entire procedure is conducted using continuous, gentle agitation.

All of the serial steps involved in bringing the blocks from formalin into paraffin and, after sectioning, bringing the mounted paraffin sections back into aqueous staining solutions, can be carried out manually, or in an automatic processor dedicated to TSE tissues. Similarly, it would be advisable to dedicate a microtome for sectioning non-formic acid treated tissue blocks, as there is no practical way to disinfect the instrument. Formic acid treated sections can be cut on a standard microtome (if possible, using a disposable knife or dedicated blade) and processed as usual. Processing fluid should be decontaminated and debris (such as wax shavings) from section cutting should be contained and disposed of by incineration (see Annex III for decontamination methods). Formic acid treated sections tend to be brittle, but show good preservation of histologic morphology.

Slides made from sections which have been treated with formic acid can be considered non-infectious. Slides made from sections that have not been treated with formic acid may also be handled without specific precautions, once the cover slip is sealed to the slide and chemically disinfected to ensure external sterility, but should be labelled as a hazardous material. These slides, if damaged, should be treated using a method described in Annex III, and destroyed.

Containers used for the storage of formalin-fixed tissues should, after secure closing, be cleaned using a method in Annex III, marked "Hazardous", and stored separately (e.g., in sealed plastic bags). When tissue is needed, the container can be removed from the bag, set upon a water-resistant disposable mat, and manipulation of the tissue confined to the mat. After the tissue is replaced, the area and container are cleaned according to methods described in Annex III, and the container put into a new plastic bag for further storage.

8.2.3 Electron microscopy

Electron microscopic examination of tissue sections is not indicated for diagnostic purposes, and is not recommended except as an investigational research tool. Preparation of specimens for electron microscopy should be performed with the same precautions as for histopathology. Electron microscopy of tissue sections poses negligible risk both to the microscope and the operator due to the very small amount of tissue deposited on a grid. An electron microscope section 0.01 micron thick x 0.1 mm x 0.05 mm contains approximately 50 pg of tissue. Even the most infectious models of the disease producing $10^{10}ID_{50}$/g of brain would result in less than 0.5 ID_{50} immobilized on the grid. Handling requires no special precautions except for disposal of such grids as infectious waste through incineration.

8.3 National and international transport of bodies

If there is a need to transport the deceased patient nationally or internationally, it will be necessary to comply with the International Civil Aviation Organization (ICAO), International Air Transport Association (IATA) Restricted Articles Regulations, and any additional requirements of the individual carriers. It should be noted that the IATA Regulations require the embalming of the body.

8.4 Undertakers and embalmers

8.4.1 General measures

Mortuary procedures may be performed on the bodies of patients who have died from CJD with a minimum of inconvenience to ensure the safety of personnel and avoid contamination of the workplace. Transportation of the unembalmed body to the mortuary should be in an sealable, impermeable plastic pouch. Ordinary contact or handling of an intact, unautopsied body does not pose a risk, and cosmetic work may be undertaken without any special precautions. If the body has undergone autopsy, care should be taken to limit contamination of the workplace by any leaking bodily fluids (especially from the cranium) when transferring the body from its transport bag to the mortuary table that has been covered with an impermeable sheet. No other precautions are required, except for embalming (see Section 8.4.2).

8.4.2 Embalming

An intact (unautopsied) body can be safely managed with only minor adjustments to the usual procedures. The body should be placed on an impermeable sheet or body pouch to avoid surface contamination from perfusion drain sites, and all drainage fluids should be collected into a stainless steel container. Perfusion sites should be closed with cyanoacrylates (super glue) and then wiped with bleach.

Embalming an autopsied or traumatized body is not encouraged, but may be safely performed when the following precautions are observed. Disposable masks, gowns, and gloves should be worn, just as is done by pathologists performing an autopsy. The body should be placed on an impermeable sheet or body pouch so that suture site leakage can be contained, and perfusion drain sites should be similarly arranged to avoid surface contamination. All drainage fluids should be collected into a stainless steel container. Perfusion and autopsy incision sites should be closed with cyanoacrylates (super glue). The entire body should be wiped down with bleach, and special care taken to ensure contact of bleach with perfusion sites and closed autopsy incisions.

At the conclusion of the perfusion procedure, the container of drainage fluids should be decontaminated by adding sodium hydroxide pellets at the rate of 40g per litre of fluid. The mixture should be stirred after a few minutes and care should be taken to avoid spillage, as the fluid will be hot. It should then be left undisturbed for at least one hour, after which it can be disposed of as for any other mortuary waste. Plastic sheets and other disposable items that have come into contact with bodily fluids should be incinerated. Mortuary working surfaces that have accidentally become contaminated should be flooded with sodium hydroxide or bleach, left undisturbed for at least one hour, then (using gloves) mopped up with absorbent disposable rags, and the surface swabbed with water sufficient to remove any residual disinfectant solution.

Non-disposable instruments and tools should be decontaminated using one of the methods recommended in Annex III. At the conclusion of the decontamination procedure, the instruments are washed with water to remove residual disinfectant fluid before drying and re-use. Sodium hydroxide or bleach can be disposed of as uninfectious (but corrosive) waste fluid.

8.5 Funerals and cremations

Relatives of the deceased may wish to view or have some final contact with the body. Superficial contact, such as touching or kissing the face, need not be discouraged,

even if an autopsy has been conducted. Interment in closed coffins does not present any significant risk of environmental contamination, and cremated remains can be considered to be sterile, as the infectious agents do not survive incineration-range temperatures (1000°C). Transport and interment are subject to local and national guidelines, and transport overseas is governed by international regulations.

8.6 Exhumations

Standard procedures are conducted according to local and national guidelines. The body should be considered as having the same infectivity as at the time of burial and the precautions used for an autopsy should be followed.

8.7 Body donation for teaching purposes

Anatomy departments should not accept, for teaching or research purposes, any body or organs from persons confirmed, suspected, or at risk for TSE, unless they have specific training or research programs for TSEs, including access to specialized equipment, procedures, appropriate containment facilities and training for managing TSE contaminated tissues. Departments should make inquiries of those responsible for donating the body, and of the medical staff involved in the care of the donor, to insure the rigorous adherence to this recommendation.

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Annex II List of Presentations

Wednesday, 24 March 1999

09.00-09.10	Welcome and introduction to the meeting	Dr Lindsay J Martinez
09.10-09.20	Selection of Chair	
09.20-09.30	Goal of meeting and opening remarks from Secretary to the meeting	
09.30-09.45	Results of the Consultation on Reagents Meeting	Chair of Reagents Meeting
09.45-10.00	Questions	ALL

Epidemiology and projections

10.00-10.30	Extent of BSE exposure worldwide	Dr Raymond Bradley
10.30-11.00	Coffee break	
11.00-11.15	vCJD epidemiology	Dr Robert Will
11.15-11.30	Predictions of the epidemic of vCJD	Dr Peter Smith
11.30-12.00	Questions on BSE, vCJD, CJD epidemiology	ALL
12.00-13.00	Lunch break	

Identification of risk

13.00-13.15	Diagnosis of CJD (iatrogenic, familial, sporadic and vCJD)	Dr Martin Zeidler
13.15-13.30	Risk assessment and ethical issues	Dr Burleigh Trevor-Deutsch
13.30-13.45	Questions	ALL
13.45-14.15	Distribution of infectivity in CJD (iatrogenic, familial, sporadic)	Dr Paul Brown
14.15-14.30	Distribution of infectivity in vCJD	Dr James Ironside
14.30-14.45	Questions on tissue, blood and organ infectivity	ALL

Decontamination procedures

14.45-15.00	Decontamination procedures	Dr David Taylor
15.00-15.25	Instruments and environment; waste disposal	Dr R. Rohwer Ms Annette Pruess
15.25-15.45	Questions on decontamination	ALL
15.45-16.15	Coffee break	
16.15-17.00	Review of day's issues and conclusions	Chair, Working Group
17.30-19.30	Cocktail party	All

Thursday, 25 March 1999

Providing care to the ill

09.00-09.30	Care givers issues	CJD Support Network, CJD Voice, Human BSE Foundation
09.30-09.45	Nursing care in the home and hospital	Ms Blair Smith-Bathgate
09.45-10.00	Questions on provision of care	ALL

Protecting healthcare and allied workers: preventing iatrogenic transmission

10.00-10.15	Nursing practice in the hospital, long term care facility and nursing home	Miss Shirley Patton
10.15-10.30	Operating theatre	Dr Martinez-Lage
10.30-11.00	Coffee break	
11.00-11.15	New results from the Australian Case-Control Study	Dr Colin Masters
11.15-11.30	Post-exposure prophylaxis for prion diseases	Dr. Sebastian Brandner
11.30-12.00	Questions on protection of HCW and patients	ALL
12.00-13.00	Lunch break	
13.00-13.30	Clinical laboratory, pathology, and autopsy procedures Questions and comments	Dr Herbert Budka
13.30-14.00	Dentistry Questions and comments	Dr J. Cleveland
14.00-14.30	Mortuary Questions and comments	Mr George Lamb
14.30-14.45	Remarks of Chairs, table draft	Chair(s), Secretariat
14.45-15.30	Group discussion	All
15.30-16.00	Coffee	
16.00-17.00	Revisions of draft document	Chair(s), Secretariat, Rapporteur(s)

Friday, 26 March 1999

09.30-10.30	Summary of previous day and revisions
10.30-11.00	Coffee break
11.00-12.00	Revision of draft document
12.00-13.00	Lunch break
13.00-14.30	Final discussions
14.30-15.00	Final recommendations to secretariat
15.00-15.30	Coffee break
15.30-16.00	Meeting of Chairs, secretariat, speakers regarding revision of document
16.00	Close

Annex III Decontamination methods for Transmissible Spongiform Encephalopathies

The safest and most unambiguous method for ensuring that there is no risk of residual infectivity on contaminated instruments and other materials is to discard and destroy them by incineration. In some healthcare situations, as described in the guidance, one of the following less effective methods may be preferred. Wherever possible, instruments and other materials subject to re-use should be kept moist between the time of exposure to infectious materials and subsequent decontamination and cleaning. If it can be done safely, removal of adherent particles through mechanical cleaning will enhance the decontamination process.

The following recommendations are based on the best available evidence at this time and are listed in order of more to less severe treatments. These recommendations may require revision if new data become available.

1. Incineration

1. Use for all disposable instruments, materials, and wastes.
2. Preferred method for all instruments exposed to high infectivity tissues.

2. Autoclave/chemical methods for heat-resistant instruments

1. Immerse in sodium hydroxide (NaOH)[20] and heat in a gravity displacement autoclave at 121°C for 30 min; clean; rinse in water and subject to routine sterilization.
2. Immerse in NaOH or sodium hypochlorite[21] for 1 hr; transfer instruments to water; heat in a gravity displacement autoclave at 121°C for 1 hr; clean and subject to routine sterilization.
3. Immerse in NaOH or sodium hypochlorite for 1 hr.; remove and rinse in water, then transfer to open pan and heat in a gravity displacement (121°C) or porous load (134°C) autoclave for 1 hr.; clean and subject to routine sterilization.
4. Immerse in NaOH and boil for 10 min at atmospheric pressure; clean, rinse in water and subject to routine sterilization.
5. Immerse in sodium hypochlorite (preferred) or NaOH (alternative) at ambient temperature for 1 hr; clean; rinse in water and subject to routine sterilization.
6. Autoclave at 134°C for 18 minutes.[22]

[20] Unless otherwise noted, the recommended concentration is 1N NaOH.

[21] Unless otherwise noted, the recommended concentration is 20 000 ppm available chlorine.

[22] In worse-case scenarios (brain tissue bake-dried on to surfaces) infectivity will be largely but not completely removed.

3. Chemical methods for surfaces and heat sensitive instruments

1. Flood with 2N NaOH or undiluted sodium hypochlorite; let stand for 1 hr.; mop up and rinse with water.
2. Where surfaces cannot tolerate NaOH or hypochlorite, thorough cleaning will remove most infectivity by dilution and some additional benefit may be derived from the use of one or another of the partially effective methods listed in Section 5.1 (Table 8).

4. Autoclave/chemical methods for dry goods

1. Small dry goods that can withstand either NaOH or sodium hypochlorite should first be immersed in one or the other solution (as described above) and then heated in a porous load autoclave at ≥ 121°C for 1 hr.
2. Bulky dry goods or dry goods of any size that cannot withstand exposure to NaOH or sodium hypochlorite should be heated in a porous load autoclave at 134°C for 1 hr.

5. Notes about autoclaving and chemicals

Gravity displacement autoclaves: Air is displaced by steam through a port in the bottom of the chamber. Gravity displacement autoclaves are designed for general decontamination and sterilization of solutions and instruments.

Porous load autoclaves: Air is exhausted by vacuum and replaced by steam. Porous load autoclaves are optimized for sterilization of clean instruments, gowns, drapes, towelling, and other dry materials required for surgery. They are not suitable for liquid sterilization.

Sodium Hydroxide (NaOH, or soda lye): Be familiar with and observe safety guidelines for working with NaOH. 1N NaOH is a solution of 40 g NaOH in 1 litre of water. 1 N NaOH readily reacts with CO_2 in air to form carbonates that neutralize NaOH and diminish its disinfective properties. 10 N NaOH solutions do not absorb CO_2, therefore, 1N NaOH working solutions should be prepared fresh for each use either from solid NaOH pellets, or by dilution of 10 N NaOH stock solutions.

Sodium hypochlorite (NaOCl solution, or bleach): Be familiar with and observe safety guidelines for working with sodium hypochlorite. Household or industrial strength bleach is sold at different concentrations in different countries, so that a standard dilution cannot be specified. Efficacy depends upon the concentration of available chlorine and should be 20 000 ppm available chlorine. One common commercial formulation is 5.25% bleach, which contains 25 000 ppm chlorine. Therefore, undiluted commercial bleach can be safely used. If solid precursors of hypochloric acid is available, then stock solution and working solutions can be prepared fresh for each use.

6. Cautions regarding hazardous materials

In all cases, hazardous materials guidelines must be consulted.

1. Personnel

NaOH is caustic but relatively slow acting at room temperature, and can be removed from skin or clothing by thorough rinsing with water. Hot NaOH is aggressively caustic, and should not be handled until cool. The hazard posed by hot NaOH explains the need to limit boiling to 10 minutes, the shortest time known to be effective.
Hypochlorite solutions continuously evolve chlorine and so must be kept tightly sealed and away from light. The amount of chlorine released during inactivation may be sufficient to create a potential respiratory hazard unless the process is carried out in a well-ventilated or isolated location.

2. Material

In principle, NaOH does not corrode stainless steel, but in practice some formulations of stainless steel can be damaged (including some used for surgical instruments). It is advisable to test a sample or consult with the manufacturer before dedicating a large number of instruments to decontamination procedures. NaOH is known to be corrosive to glass and aluminum. Hypochlorite does not corrode glass or aluminum and has also been shown to be an effective sterilizing agent; it is, however, corrosive both to stainless steel and to autoclaves and (unlike NaOH) cannot be used as an instrument bath in the autoclave. If hypochlorite is used to clean or soak an instrument, it must be completely rinsed from the surfaces before autoclaving. Other decontamination methods may need testing, or consultation with the manufacturer to verify their effect on the instrument.

Annex IV Management of healthy 'at risk' individuals

Tissue recipients

The consultation felt that the risk from *recipients of dura mater, cornea transplants* and *human pituitary hormones,* and *from persons who have undergone neurosurgical procedures,* is no longer sufficient to warrant classifying this population as a risk for transmitting TSEs, except under conditions where there could be exposure to their high infectivity tissues (see Section 2.4.2). The consultants considered that appropriate control measures have immensely reduced or eliminated exposure to contaminated dura mater and pituitary hormones, and noted that there are only three reports of TSE transmission through cornea transplantation, and six reports (all before 1980) of transmission via neurosurgical instruments. In addition, it was recognized that recipients of dura mater are largely unaware of the fact, making identification of many of the dura mater recipients unlikely.

Countries not applying appropriate control measures cannot assume similarly low levels of current risk among tissue recipients.

Familial Transmissible Spongiform Encephalopathies

Consensus was not reached as to whether asymptomatic persons at risk for *familial TSE* should be classified as 'at risk' when determining appropriate infection control levels. It was argued that the identification of familial risk among asymptomatic people would confer a lifetime requirement for high-level infection control for a transmission risk that remains only hypothetical. Discrimination against such persons and legal implications regarding their access to insurance, employment and healthcare was described by several participants in the consultation, and it was proposed that such discrimination would inevitably lead to a harm which exceeded any evidence of risk posed by them to others.

Others argued that if a familial risk were identified, then more stringent levels of infection control could be adopted even in the absence of firm evidence of risk, particularly during procedures involving *high infectivity tissues*. All consultants agreed that persons 'at risk' for familial TSE should not be denied access to treatment or surgical procedures, particularly given the range of decontamination options available. Scientific resolution of these issues was impossible due to a lack of precise information about tissue infectivity during the pre-clinical phase of human disease, and the consultants emphasized the need to study any available tissues (including blood) from mutation-positive, but still asymptomatic, members of TSE families.

Annex V Management of individuals with confirmed or suspected variant Creutzfeldt-Jakob Disease

The TSE agent causing vCJD has shown certain differences from that of sporadic CJD, including the detection of prion protein (PrP) in a range of lymphoreticular tissues. Patients with vCJD might therefore pose a greater risk of transmitting iatrogenic infections than sporadic CJD. However, this hypothetical risk has to be balanced against the real danger of stigmatizing patients and causing distress and anxiety to the patient's relatives by the introduction of rigorous and possibly unnecessary infection control procedures in general patient care.

On current evidence, the infection control procedures in nursing care settings for sporadic CJD may be applied to cases of vCJD without the need for additional precautions, although a more conservative approach may be taken for interventions involving surgical procedures, or when handling tissues and body fluids in the laboratory. See Section 6.6 (Table 9) for measures that have been recommended for high infectivity tissues in patients with other forms of TSE, that could be applied to all tissues of persons with vCJD. It is noted that considerable safety is afforded through the measures described in Section 6 and Annex III, and that no person should be denied a diagnostic test given the efficacy of the recommended measures. Comparative tissue risks for vCJD and a case definition of possible vCJD cases will need to be redefined as further research findings emerge. If vCJD is suspected, consultation with persons expert in this disease, such as The Edinburgh CJD Surveillance Unit, Western General Hospital, United Kingdom, is recommended.

WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies

This document provides an authoritative guide to procedures and precautions needed to prevent iatrogenic and nosocomial exposure to transmissible spongiform encephalopathies (TSEs) in hospitals, health care facilities, and laboratories. Prepared by an international group of 32 leading experts, the guidelines respond to the unusual resistance of TSE agents to conventional chemical and physical methods of decontamination and the corresponding need for special precautions. Areas of patient care and categories of interventions, tissues, instruments, and wastes that do not require special precautions are also clearly indicated.

In issuing these guidelines, WHO aims to help medical officers, specialists in infection control, care-givers, and laboratory workers reduce the risks of TSE transmission to negligible levels. With this goal in mind, the guidelines provide a logical framework for determining levels of risk and knowing when departures from standard procedures for infection control are required.

Specific recommendations are set out in tables and explanatory text covering patient care, occupational injury, laboratory investigations, decontamination procedures, waste disposal, and precautions after death. Adherence to these procedures should ensure a high level of safety. As the document repeatedly emphasizes, no TSE patient should be denied admission to a health facility, kept in isolation, or deprived of any procedure.

Further practical advice is provided in a series of annexes, which give exact instructions for recommended decontamination methods and discuss the management of healthy "at risk" individuals and individuals with confirmed or suspected variant Creutzfeldt-Jakob disease.

Chemical denaturation

Properties of the Scrapie Prion Protein: Quantitative Analysis of Protease Resistance†

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ABSTRACT: The disease-specific isoform of the prion protein (PrP^{Sc}) is an essential part of the infectious particle which causes spongiform degeneration in various mammalian species. PrP^{Sc} differs from PrP of normal animals (PrP^{C}) by its relative protease resistance. The physical nature of this difference is still unknown. We analyzed the protease resistance of PrP^{Sc} quantitatively using an enzyme-linked immunofiltration assay. PrP^{Sc} was rendered completely protease-sensitive at alkaline pH or in >1.5 M guanidinium thiocyanate (GdnSCN). Denaturation in 4 M GdnSCN completely abolished the protease resistance of PrP^{Sc} within 15 min, while denaturation in 7.2 M urea showed a slower time course. In the presence of ethanol, PrP^{Sc} was protected from denaturation by GdnSCN or alkaline pH. Denaturation curves were used to calculate the free energy (ΔG_D) as a function of different denaturant concentrations. Linear regression of ΔG_D values was used to extrapolate the free energy in the absence of denaturants (ΔG_{H_2O}), yielding similar values ($\Delta G_{H_2O,GdnSCN} = -2.3$ kcal/mol; $\Delta G_{H_2O,urea} = -3.1$ kcal/mol). The linear relationship between ΔG_D and the denaturant concentration is suggestive of a two-state model involving the conformational change of a single protein domain. This is also reflected in the small number of side chains (11.6) additionally exposed to the solvent upon conversion of PrP^{Sc} to its protease-sensitive isoform. Our results suggest that only minor rearrangements of the structure of PrP are needed to abolish the protease resistance of PrP^{Sc}.

The disease-specific isoform of the prion protein (PrP^{Sc}) is part of the infectious particle causing scrapie in sheep, bovine spongiform encephalopathy in cattle, or Creutzfeldt–Jakob disease in humans. PrP^{Sc} differs from the normal cellular prion protein (PrP^{C}) by its relative protease resistance (Bolton et al., 1982; Oesch et al., 1985). The molecular changes leading to this difference in physicochemical properties are unknown. PrP^{Sc} to date has never been separated from infectivity (McKinley et al., 1983; Gabizon et al., 1988; DeArmond et al., 1987; Sklaviadis et al., 1989). This led to the proposal that the infectious particle would be composed of specifically altered PrP molecules (Prusiner, 1991).

Various hypotheses regarding these alterations have been disproved. The amino acid sequence of PrP^{Sc} is identical to that predicted from cDNA or genomic nucleic acid sequences (Oesch et al., 1985; Chesebro et al., 1985; Stahl et al., 1993), and the infectious particle does not encode an altered PrP gene (Oesch et al., 1985). In cell culture, PrP^{C} is converted into PrP^{Sc} posttranslationally (Caughey et al., 1989; Borchelt et al., 1990). Inhibition of asparagine-linked glycosylation did not prevent the synthesis of protease-resistant PrP^{Sc} (Taraboulos et al., 1990). No difference in covalent modifications of PrP^{Sc} and PrP^{C} was observed using mass spectrometry (Stahl et al., 1991, 1993). As an alternative, it was proposed that PrP^{C} might interact with a second component (most likely a nucleic acid), leading to a modification of the properties of PrP (Bruce & Dickinson, 1987; Hope et al., 1986; Weissmann, 1991b). Prion-specific nucleic acids are still elusive (Oesch et al., 1988; Kellings et al., 1992), while two peptides have been observed reproducibly in PrP^{Sc} preparations (Stahl et al., 1993). These peptides may be indicative of other proteinaceous components present in prion preparations such as nucleic acid binding proteins (Sklaviadis et al., 1993) or proteoglycans which have been colocalized with PrP amyloid deposits (Nochlin et al., 1989; Snow et al., 1990). However, the role of these compounds in scrapie remains to be clarified.

The lack of a molecular explanation for the observed differences between PrP^{Sc} and PrP^{C} led to the proposal that they differ in conformation (Basler et al., 1986; Stahl & Prusiner, 1991; Stahl et al., 1993). By infrared spectroscopy, a high content of β-sheet structure was detected for PrP 27–30, the protease-resistant core of PrP^{Sc} (Caughey et al., 1991; Gasset et al., 1993). A reduced content of β-sheet structure is found in PrP^{C}, suggesting differences in secondary structure between the two isoforms of PrP (Pan et al., 1993).

The relative protease resistance of PrP^{Sc} is currently the only way to distinguish the two forms of PrP (Oesch et al., 1985). Serban et al. (1990) developed an assay to detect PrP^{Sc} that involved immobilization of proteins on nitrocellulose followed by protease digestion, denaturation, and immunodetection with monoclonal antibodies. To quantitate the amount of PrP^{Sc}, we have refined an enzyme-linked immunofiltration assay (ELIFA) for PrP^{Sc} (Prusiner et al., 1990). Protease resistance of PrP^{Sc} was reduced at basic pH or by incubation in chaotropic reagents while the presence of ethanol protected PrP^{Sc} from denaturation. We have also analyzed the thermodynamic parameters for the conversion of PrP^{Sc} to a protease-sensitive form, suggesting only a minor difference in free energy between the two isoforms.

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MATERIALS AND METHODS

Materials. Scrapie brain homogenates, purified PrP 27–30, and 13A5 monoclonal antibodies were a kind gift of Dr. S. B. Prusiner, University of California, San Francisco. Avidin and biotin were purchased from Boehringer Mannheim. Proteinase K and human serum albumin (HSA) were from Sigma. Streptavidin coupled to peroxidase and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate for peroxidase were from Kirkegaard and Perry Laboratories, Gaitherburg, MD; other chemicals were purchased from Fluka, Buchs, Switzerland. Nitrocellulose (0.2 μm) and the ELIFA blotting apparatus were from Pierce.

Standard ELIFA Procedure. Samples were applied to nitrocellulose filters (0.2 μm) in the ELIFA apparatus in a 100-μL final volume containing 1 M guanidinium thiocyanate (GdnSCN) and 5 mM DTT. Appropriate dilutions (1:200 to 1:2000) were applied. Wells were rinsed with 100 μL of sample dilution buffer (1 M GdnSCN, 0.01% HSA, 150 mM NaCl, and 10 mM $NaPO_4$, pH 7.4). Filters were taken out of the block, washed in water, dried at room temperature (RT) for 30 min, and then wetted in 150 mM NaCl and 10 mM $NaPO_4$, pH 7.4 (PBS), followed by incubation in 1% KOH for 5 min. Filters were then washed in PBS followed by 0.5% HSA in 0.05% Tween 20 and PBS (PBST) for 30 min. All incubations were done at room temperature unless otherwise indicated, and filters were washed 3 times for 5 min with PBST between steps. Biotin in the applied samples was blocked by incubation in 25 μg/mL avidin/PBST followed by 2 μg/mL *d*-biotin/PBST for 30 min each. Biotinylated monoclonal antibody 13A5 (Prusiner et al., 1990) was incubated in PBST for 14–18 h at 4 °C on a rocking platform. After being washed in PBST, filters were incubated in 0.1 μg/mL streptavidin coupled to peroxidase for 1 h. For detection, filters were placed back in the ELIFA block and the substrate (0.2 mg/mL TMB, 0.01% H_2O_2) was pulled through into an ELISA plate placed underneath. Color was stabilized by addition of 100 μL of 1 M H_3PO_4. OD_{450} versus OD_{600} was determined in an SLT 400 ATX ELISA reader (SLT Labinstruments, Gröding, Austria).

PrP Standards. Purified PrP 27–30 was denatured in 4 M GdnSCN for 1 h at 37 °C and then diluted to 1 M GdnSCN and stored at a concentration of 2 μg/mL at −70 °C. The amount of PrP was determined by amino acid analysis as described (Stahl et al., 1990). A standard curve for various amounts of PrP (0.1–2 ng/well) was generated using GdnSCN-denatured PrP 27–30. The lower limit of detection was 0.1 ng of purified PrP 27–30. To control for variability, each filter contained duplicates of PrP standards. The parameters for the standard curve were obtained by linear regression analysis and then used for the calculation of the PrP quantity in test samples. The correlation coefficient of the standard curves was usually greater than 0.98.

Detection of PrP^{Sc}. Ten percent brain homogenates from terminally ill, scrapie-infected hamsters were centrifuged at 1000*g* for 20 min. Pelleted nuclei were discarded. The supernatant (cleared scrapie brain homogenate, cSBH) was stored at −70 °C in aliquots to be used in further experiments. The amount of PrP^{Sc} in a given sample was determined as follows: samples were digested with 50 μg/mL proteinase K for 30 min at 37 °C. Proteinase K was inactivated by the addition of 7.5 mM PMSF. Samples then were denatured in 4 M GdnSCN for 1 h at 37 °C, diluted to 1 M GdnSCN, and applied to nitrocellulose as described above. As a control, half of the native sample was immediately denatured in 4 M GdnSCN, diluted to 1 M GdnSCN, and then either digested with proteinase K, yielding the background of the particular assay, or applied untreated, giving the total amount of PrP.

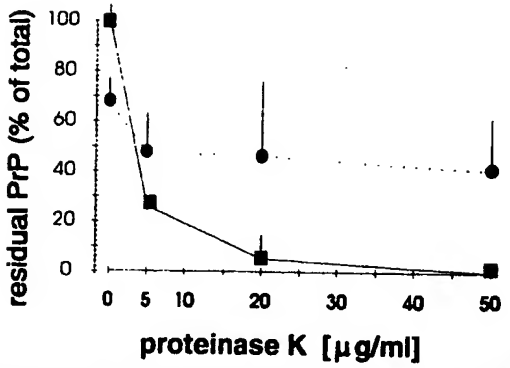

FIGURE 1: Sensitivity of PrP^C, PrP^{Sc}, and denatured PrP to digestion with proteinase K. Native (●) or 4 M GdnSCN denatured (■) brain homogenates were treated with the indicated concentrations of proteinase K. PrP is expressed relative to the amount of total PrP in denatured, untreated homogenates.

Treatment of cSBH with GdnSCN, Urea, Different pH, and Ethanol. cSBH (1/10th volume) was mixed with 6 M GdnSCN or 8 M urea to give a final 10-fold dilution with the appropriate denaturant concentration. Samples were incubated at 37 °C for 1 h and diluted 4-fold, and residual PrP^{Sc} was determined. The buffers for the different pH's were as follows: pH 2–6, 100 mM citric acid adjusted with NaOH; pH 7, 7.4, 8, and 8.5, 100 mM Tris base adjusted with HCl; pH 9–11, 100 mM Na_2CO_3 adjusted with HCl. Incubation at the various pH's was done at 37 °C for 1 h. For treatment with ethanol, incubation buffers were mixed to 9/10 of the final volume followed by the addition of 0.1 volume cSBH. All samples were incubated at 37 °C for 1 h followed by determination of PrP^{Sc}.

RESULTS

Enzyme-Linked Immunofiltration Assay (ELIFA) for the Scrapie-Specific Isoform of Prion Protein. PrP^C is defined as the protease-sensitive form of PrP, whereas PrP^{Sc} is partially protease-resistant. PrP^C is readily detected, but native, protease-resistant PrP^{Sc} is not recognized by antibodies. Therefore, PrP^{Sc} needs to be denatured prior to immunodetection (Kitamoto et al., 1987; Serban et al., 1990; Prusiner et al., 1990). We applied an ELIFA system to quantitate PrP. Briefly, protein samples were applied to nitrocellulose using a slot-blot apparatus. Membranes then were incubated with biotinylated anti-PrP monoclonal antibodies (13A5; Prusiner et al., 1990) followed by streptavidin coupled to peroxidase. For quantitation, the membrane was placed back on the slot-blot apparatus and soluble reaction product was pulled into ELISA plates placed underneath. Subsequently, color development was quantitated in a conventional ELISA reader.

The distinction between PrP^C and PrP^{Sc} was made by the digestion of samples with various amounts of proteinase K (Figure 1). Subsequently, proteinase K was blocked with PMSF, and the sample was denatured with 4 M GdnSCN and then used in the ELIFA procedure described above. PrP^{Sc} was measured relative to the total amount of PrP present in the same sample without proteinase K digestion. About 50% of total PrP could not be digested with proteinase K concentrations up to 50 μg/mL (Figure 1, circles). As a control, extracts were first denatured in 4 M GdnSCN and then treated with proteinase K, resulting in complete digestion of all PrP (Figure 1, squares). Surprisingly, the undenatured sample contained less PrP at 0 μg/mL proteinase K than the

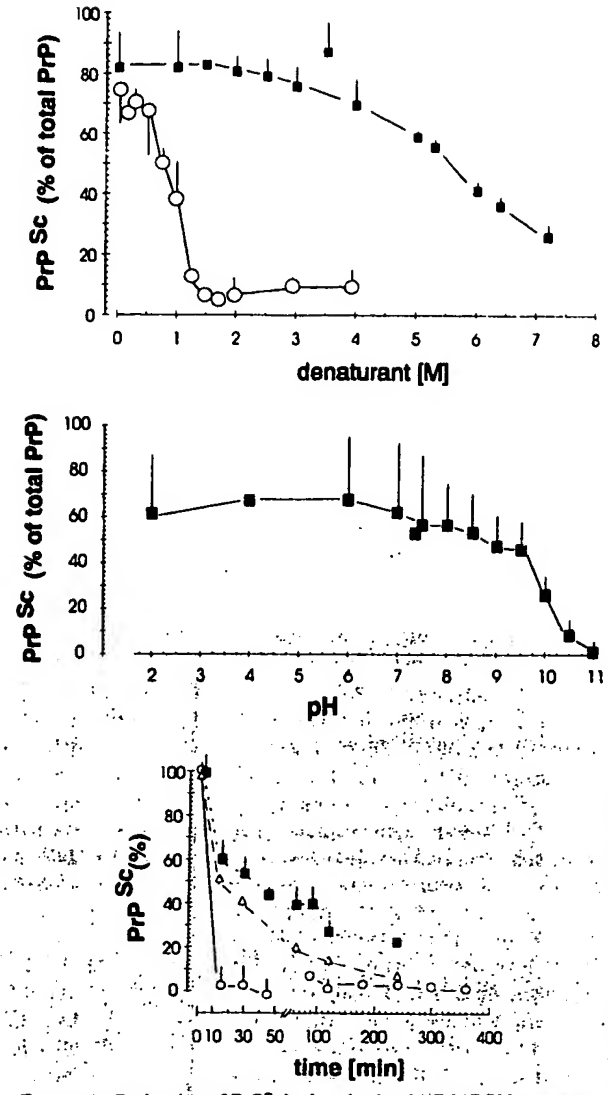

FIGURE 2: Reduction of PrP^{Sc} by incubation in GdnSCN or urea or at different pH's. (A, top) Cleared brain homogenates were incubated in various concentrations of GdnSCN (○) or urea (■) for 1 h at 37 °C followed by determination of residual protease-resistant PrP. (B, middle) Residual PrP^{Sc} after incubation at the indicated pH. (C, bottom) Time course of denaturation of PrP^{Sc} by 4 M GdnSCN (○), by 7.2 M urea (■), or at pH 11 (▲). PrP^{Sc} at 0 min was set at 100%.

GdnSCN-denatured sample. We suspect that endogenous proteases degrade PrP^{C} in the native sample during the incubation at 37 °C, and that these proteases may be inactivated in the samples first denatured in GdnSCN.

Treatments Rendering PrP^{Sc} Protease-Sensitive., Brain extracts containing PrP^{Sc} were incubated in various concentrations of GdnSCN or urea (Figure 2A) or at different pH's (Figure 2B). Incubation in 1.5 M GdnSCN rendered PrP^{Sc} completely protease-sensitive. Urea at the highest concentration (7.2 M) reduced PrP^{Sc} to approx. 25% (Figure 2A). The same result was observed when the samples were incubated at 37 or 4 °C (results not shown). As a control for proteinase K activity, denatured PrP^{Sc} was treated in parallel under the same conditions, resulting in complete digestion of all PrP (results not shown). The transition midpoints for conversion of PrP^{Sc} to protease-sensitive PrP were at 0.87 M GdnSCN and 5.8 M urea, respectively.

Changes in the protease resistance of PrP^{Sc} were also measured after incubation at various pH's (Figure 2B). It has been suggested that PrP^{Sc} undergoes pH-dependent conformational changes (Gasset et al., 1993). The amount of PrP^{Sc} was unchanged following incubation at various pH's between 2 and 9.5, while it decreased at pH 10. PrP^{Sc} was no longer detectable after incubation at pH 11.

The time course of denaturation was analyzed for treatment with GdnSCN (4 M) or urea (7.2 M) and at pH 11 (Figure 2C). Denaturation of PrP^{Sc} was complete after 15 min with GdnSCN, while denaturation by urea was slower with a half-maximal inactivation at 30 min. The time course of denaturation at pH 11 was intermediate between those with GdnSCN and urea denaturation; i.e., half-maximal denaturation was observed at 15 min (Figure 2C). Incubation at 37, 20, or 4 °C gave the same results (not shown). Gasset et al. (1993) found that an irreversible change in conformation occurs at alkaline pH. This conformational change can therefore be correlated with increased solubility of PrP^{Sc}, a loss in infectivity, and a loss of protease resistance (Gasset et al., 1993; Figure 2B).

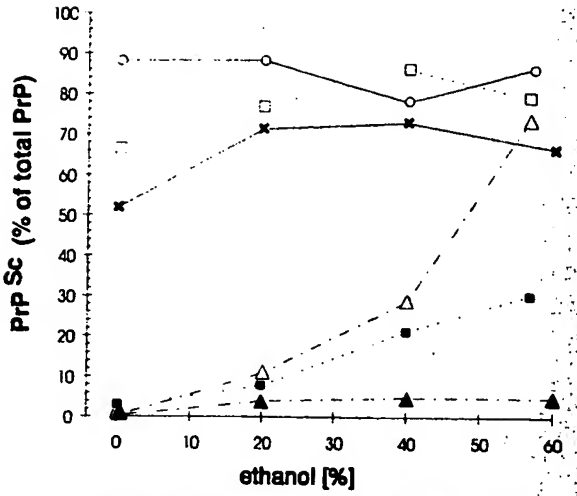

FIGURE 3: Effect of ethanol on denaturation of PrP^{Sc}. Brain extracts were incubated at pH 7 (○), pH 2 (□), pH 11 (■), 6.9 M urea (×), or 4 M GdnSCN (△,▲) in the presence of the indicated concentrations of ethanol. For the incubations in GdnSCN, ethanol was added either simultaneously (△) or 15 min after the GdnSCN (▲).

Protection of PrP^{Sc} by the Presence of Ethanol. Ethanol is used commonly to dehydrate proteinaceous samples, often leading to denaturation (Touati et al., 1992; Pan & Briggs, 1992; Ramalingam & Bello, 1993; Fan et al., 1993; Wang et al., 1993). We were interested to see whether dehydration of PrP^{Sc} would facilitate denaturation. PrP^{Sc} was incubated in increasing concentrations of ethanol. Under standard conditions (pH 7) we did not find any change in PrP^{Sc} levels with concentrations up to 60% ethanol (Figure 3, open squares). When we used increasing concentrations of ethanol in combination with 6.9 M urea, or pH 2 we observed a slight increase in PrP^{Sc} indicating that PrP^{Sc} might be protected from denaturation in the presence of ethanol. This effect is much more pronounced upon treatment with 4 M GdnSCN: in the absence of ethanol all PrP^{Sc} is converted into the protease-sensitive form, while in the presence of 60% ethanol the denaturing effect of GdnSCN is completely neutralized (open triangles). When GdnSCN was added first followed by the addition of various concentrations of ethanol, we did not find any recovery of PrP^{Sc}, indicating that ethanol is unable to restore protease resistance (closed triangles). Similarly, treatment at pH 11 reduced PrP^{Sc} to background levels in the absence of ethanol but was less effective at higher concentrations of ethanol.

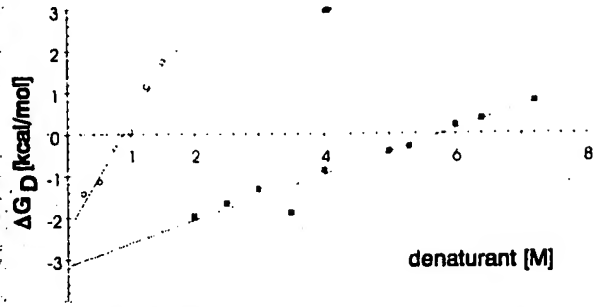

FIGURE 4: Dependence of the free energy, ΔG_D, on denaturant concentration. The denaturation curves from Figure 2 were used to calculate ΔG_D as a function of the GdnSCN (○) or urea concentration (■) as described in Results. The lines were determined by linear regression.

Transition of PrP^{Sc} to the Protease-Sensitive Isoform Requires Only Small Changes in Secondary Structure. The unfolding of proteins in denaturants has been studied extensively using spectroscopic methods (Dill & Shortle, 1991; Zerovnik et al., 1992; Dyer et al., 1992; Staniforth et al., 1993; Pappa & Cass, 1993; Ahmad et al., 1992). The resulting denaturation curves are analyzed for the number of transitions which indicate unfolding of independent domains. In addition, the free energy and the number of additionally exposed amino acid side chains can be derived. We assumed that the secondary or tertiary structure of PrP would be the cause of protease resistance. Therefore, denaturation curves were measured using the protease sensitivity of PrP as a parameter to follow the changes in conformation (Figure 2). Ideally, we would be able to derive information about the nature of the scrapie-specific part in PrP^{Sc}. We were first interested in the change of free energy (ΔG_D) upon denaturation of PrP^{Sc} as a function of the concentration of GdnSCN or urea. ΔG_D is calculated according to the relationship

$$\Delta G = -RT \ln K \qquad (1)$$

where K is the ratio of protease-resistant to protease-sensitive PrP, which has been determined experimentally (Figure 2). The ΔG_D values were plotted versus the denaturant concentration itself (Figure 4). Linear regression analysis revealed correlation coeffients of $r = 0.988$ (GdnSCN) and $r = 0.971$ (urea) indicative of a single transition midpoint for both denaturants. Thus, the conversion of PrP^{Sc} to the protease-sensitive form of PrP appears to depend on the conformational change of a single domain. Extrapolation of ΔG_D values to a value in the absence of denaturant (ΔG_{H_2O}) yielded similar ΔG_{H_2O} values for both denaturants ($\Delta G_{H_2O,GdnSCN} = -2.3$ kcal/mol; $\Delta G_{H_2O,urea} = -3.1$ kcal/mol; Figure 4).

Assuming that most of the energy requirement would be due to exposure of previously buried amino acid side chains (Staniforth et al., 1993), the number of newly exposed side chains (n_e) was calculated according to the equation

$$\Delta G_D = \Delta G_{H_2O} + n_e \Delta G_{s,d} \qquad (2)$$

where $\Delta G_{s,d}$ is the difference in solvation energy required to expose an average side chain as a function of the denaturant concentration. $\Delta G_{s,d}$ can be calculated according to the formula

$$\Delta G_{s,d} = (\sum \Delta G_{si} n_i)/n \qquad (3)$$

where ΔG_{si} is the free energy required to expose a particular side chain, n_i is the number of side chains of type i in the solvent-excluded core, and n is the total number of side chains in the core. ΔG_{si} values for different concentrations of urea were taken from Staniforth et al. (1993). The exposure of side chains in PrP^{Sc} to the solvent is unknown; hence we have applied average values determined from 55 proteins in the Brookhaven Protein Data Bank. It has been shown that these values can be applied to proteins with unknown secondary structure (Staniforth et al., 1993). These calculations were applied to the protease-resistant core of PrP^{Sc} (amino acids 89–231). Solving eqs 2 and 3 for concentrations of 4–7.2 M urea, we calculated the number of additionally exposed amino acid side chains (n_e) to be 11.6 ± 0.49. Thus, the conversion of PrP^{Sc} to its protease-sensitive form seemed to require only a minor change in conformation.

DISCUSSION

The protease resistance of PrP^{Sc} is the major property which is specific for the infectious particle. The nature of the presumed alterations is defined by exclusion: (1) There are no substitutions in the amino acid sequence (Oesch et al., 1985; Hope et al., 1986; Basler et al., 1986; Stahl et al., 1993). (2) No PrP^{Sc}-specific posttranslational modifications have been found; both forms of PrP have N-linked carbohydrates and a glycolipid anchor (Haraguchi et al., 1989; Stahl et al., 1987). It is not known whether modifications of two arginines at the N-terminus of PrP^{Sc} are also found in PrP^{C} (Hope et al., 1986; Stahl et al., 1993). (3) No molecules other than PrP^{Sc} are linked to infectivity (Bolton et al., 1982; Hope et al., 1986; Gabizon et al., 1987). Therefore, it was proposed that PrP^{Sc} and PrP^{C} differ in their conformation (Basler et al., 1986; Stahl et al., 1993).

Taking these previous findings as a basis for our analysis of denaturation curves of PrP^{Sc}, we assumed that the incubation in denaturants would lead to an alteration of PrP^{Sc} conformation. Our calculations revealed a difference in free energy of 2.3–3.1 kcal/mol and the exposure of 11.6 additional amino acid side chains for the transition from PrP^{Sc} to the protease-sensitive form of PrP. The relatively small change in free energy and additionally solvent-exposed amino acid side chains makes it unlikely that there is a complete unfolding of the polypeptide chain but rather the unfolding of a specific domain. Unfolding of other proteins typically exposes 40–80 amino acid side chains per domain; i.e., phosphoglycerate kinase (PGK) has three transitions with ΔG_{H_2O} values of 3.8, 10.8, and 8.2 kcal/mol and exposure of 55.5, 84.8, and 51 additional side chains, respectively (Staniforth et al., 1993). This represents a total of 191.3 additionally exposed side chains, which is equivalent to the calculated number of solvent-excluded amino acid side chains of the native molecule. Therefore, all the side chains are exposed to the solvent in the fully denatured protein. Comparing these values to the denaturation parameters of PrP^{Sc}, we conclude that only minor perturbations of the structure of PrP lead to the conversion of PrP^{Sc} to protease-sensitive PrP. The secondary structure of PrP^{Sc} and PrP^{C} has also been investigated by infrared spectroscopy (Caughey et al., 1991; Gasset et al., 1993; Pan et al., 1993). A high proportion of β-sheet structure was present in purified PrP^{Sc}, while the secondary structure of PrP^{C} appears to be predominantly α-helical (Pan et al., 1993). It will therefore be important to analyze the folding of specific domains of PrP by independent methods.

What fraction of the energy difference between PrP^{Sc} and protease-sensitive PrP can be attributed to the scrapie-specific part of PrP^{Sc}? Our calculations above suggest that a specific domain of PrP is folded differently in PrP^{Sc} and PrP^{C} and that

this difference in folding may be responsible for the protease resistance of PrP^{Sc}. ΔG values for the denaturation of this domain in PrP^{C} are not available; however, the region between amino acids 106 and 126 spontaneously forms amyloid which is partially protease resistant (Gasset et al., 1992; Selvaggini et al., 1993). The protease-resistant conformation of this region therefore seems to be energetically favored. The same domain in PrP^{C} may be unable to adopt this conformation. PrP^{Sc} may therefore be the energetically more stable form; however, the formation of PrP^{Sc} would normally be prevented due to a higher activation energy (Weissmann, 1991a).

Alternative explanations for the protease resistance of PrP^{Sc} suggest an association of PrP with other cellular (or viral) components such as nucleic acids or proteoglycans (Snow & Wight, 1989; Snow et al., 1989; Czub et al., 1988; Diringer et al., 1991; Sklaviadis et al., 1993). It also has been suggested that nucleation-dependent polymerization of PrP is directly involved in the pathogenic mechanism of scrapie (Jarrett & Lansbury, 1993). PrP^{Sc} would be in an equilibrium between an aggregated form (amyloid) and a soluble form. If the aggregation was the reason for the protease resistance of PrP^{Sc}, we could interpret the difference in free energy, ΔG_{H_2O}, as a function of the equilibrium between free and amyloid-bound PrP^{Sc}. Under normal conditions (37 °C) this equilibrium would be on the side of the amyloid by a factor of 40 or higher ($\ln([\text{amyloid}]/[\text{free}]) = -\Delta G/RT$). The number of additionally exposed amino acids could then be interpreted as the size of the contact area between PrP molecules. These conclusions are particularly interesting in view of mutations in the prion protein linked to the occurrence of prion diseases in humans. Mice transgenic with mutant PrP (Pro → Leu at codon 102; Hsiao et al., 1990) did not produce significant amounts of protease-resistant PrP even though these animals had spontaneous prion disease. Mutant prion proteins may not be driven into a different conformation but rather might change more easily between conformations or, in the latter model, between the free and amyloid states.

We have reported here that ethanol stabilized PrP^{Sc} against denaturation by GdnSCN (Figure 3). In other proteins (β-globin, apocytochrome *c*, monellin, ubiquitin) alcohols restrict the conformational flexibility (Acharya et al., 1992; Fan et al., 1993; Pan & Briggs, 1992). This leads to a decreased susceptibility of β-globin, apocytochrome *c*, and the streptococcal PepM49 protein to proteolysis (Acharya et al., 1992). The gradual effect of increasing concentrations of ethanol suggests that the observed protease resistance is not due to precipitation of proteins. However, the aggregated state of PrP^{Sc} may be stabilized by the presence of ethanol. Alternatively, alcohols like ethanol, trifluoroethanol, or *n*-propanol induce α-helical structures. The presence of 50% ethanol induces the change of monellin from all β-sheet to α-helix (Fan et al., 1993). We would therefore have expected a destabilization of PrP^{Sc}, which is known to have a large content of β-sheet structure (Caughey et al., 1991; Gasset et al., 1993), while PrP^{C} is predominantly α-helical (Pan et al., 1993). Since we observe a stabilization of PrP^{Sc}, we cannot easily reconcile these findings with structural predictions.

The comparison of conditions to inactivate prions and to reduce protease resistance of PrP^{Sc} shows an excellent correlation. Infectivity and the protease resistance of PrP^{Sc} are reduced at alkaline pH and by urea (Gasset et al., 1993; Prusiner et al., 1981, 1993; Mould et al., 1965). Denaturation of prions by 1 M GdnSCN for 1 h did not reduce infectivity, while in 2 M GdnSCN infectivity was reduced 1000-fold (Prusiner et al., 1993). This correlates quite well with the reduction in protease resistance of PrP^{Sc} observed in our experiments even though we would have predicted a loss of ~50% of infectivity at 1 M GdnSCN after 1 h. This may be due to the relative imprecision of the infectivity assay, where a reduction by a factor of 2 is very difficult to determine.

CONCLUSIONS

We have characterized the nature of the protease resistance of PrP^{Sc} through the analysis of unfolding induced by GdnSCN or urea. The most striking result is the apparently low free energy required to convert PrP^{Sc} into a protease-sensitive form. Only a small number of amino acids are exposed additionally upon destruction of protease resistance. These seemingly minor requirements to destroy the protease resistance of PrP^{Sc} contrast with the high stability of prions against procedures which readily inactivate other infectious particles and may reflect the proteinaceous rather than viral nature of the infectious particles causing scrapie, bovine spongiform encephalopathy, or Creutzfeldt–Jakob disease.

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REFERENCES

Acharya, A. S., Iyer, K. S., Sahni, G., Khandke, K. M., & Manjula, B. N. (1992) *J. Protein Chem. 11*, 527–538.

Ahmad, F., Yadav, S., & Taneja, S. (1992) *Biochem. J. 287*, 481–485.

Basler, K., Oesch, B., Scott, M., Westaway, D., Walchli, M., Groth, D. F., McKinley, M. P., Prusiner, S. B., & Weissmann, C. (1986) *Cell 46*, 417–428.

Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1982) *Science 218*, 1309–1311.

Borchelt, D. R., Scott, M., Taraboulos, A., Stahl, N., & Prusiner, S. B. (1990) *J. Cell Biol. 110*, 743–752.

Bruce, M. E., & Dickinson, A. G. (1987) *J. Gen. Virol. 68*, 79–89.

Caughey, B., Race, R. E., Ernst, D., Buchmeier, M. J., & Chesebro, B. (1989) *J. Virol. 63*, 175–181.

Caughey, B. W., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., & Caughey, W. S. (1991) *Biochemistry 30*, 7672–7680.

Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., & Keith, J. M. (1985) *Nature 315*, 331–333.

Czub, M., Braig, H. R., & Diringer, H. (1988) *J. Gen. Virol. 69*, 1753–1756.

DeArmond, S. J., Mobley, W. C., DeMott, D. L., Barry, R. A., Beckstead, J. H., & Prusiner, S. B. (1987) *Neurology 37*, 1271–1280.

Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795–825.

Diringer, H., Blode, H., & Oberdieck, U. (1991) *Arch. Virol. 118*, 127–131.

Dyer, J. M., Nelson, J. W., & Murai, N. (1992) *J. Protein Chem. 11*, 281–288.

Fan, P., Bracken, C., & Baum, J. (1993) *Biochemistry 32*, 1573–1582.

Gabizon, R., McKinley, M. P., & Prusiner, S. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4017–4021.

Gabizon, R., McKinley, M. P., Groth, D., & Prusiner, S. B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6617–6621.

Gasset, M., Baldwin, M. A., Lloyd, D. H., Gabriel, J. M., Holtzman, D. M., Cohen, F., Fletterick, R., & Prusiner, S. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10940–10944.

Gasset, M., Baldwin, M. A., Fletterick, R. J., & Prusiner, S. B. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1–5.
Haraguchi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarentino, A., Borchelt, D. R., Teplow, D., Hood, L., Burlingame, A., & Prusiner, S. B. (1989) *Arch. Biochem. Biophys. 274*, 1–13.
Hope, J., Morton, L. J., Farquhar, C. F., Multhaup, G., Beyreuther, K., & Kimberlin, R. H. (1986) *EMBO J. 5*, 2591–2597.
Hsiao, K. K., Scott, M., Foster, D., Groth, D. F., DeArmond, S. J., & Prusiner, S. B. (1990) *Science 250*, 1587–1590.
Jarrett, J. T., & Lansbury, P. T. (1993) *Cell 73*, 1055–1058.
Kellings, K., Meyer, N., Mirenda, C., Prusiner, S. B., & Riesner, D. (1992) *J. Gen. Virol. 73*, 1025–1029.
Kitamoto, T., Ogomori, K., Tateishi, J., & Prusiner, S. B. (1987) *Lab. Invest. 57*, 230–236.
McKinley, M. P., Bolton, D. C., & Prusiner, S. B. (1983) *Cell 35*, 57–62.
Mould, D. L., Dawson, A. M., & Smith, W. (1965) *Res. Vet. Sci. 6*, 151–154.
Nochlin, D., Sumi, S. M., Bird, T. D., Snow, A. D., Leventhal, C. M., Beyreuther, K., & Masters, C. L. (1989) *Neurology 39*, 910–918.
Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B., & Weissmann, C. (1985) *Cell 40*, 735–746.
Oesch, B., Groth, D. F., Prusiner, S. B., & Weissmann, C. (1988) *Ciba Found. Symp. 135*, 209–223.
Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., & Prusiner, S. B. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10962–10966.
Pan, Y., & Briggs, M. S. (1992) *Biochemistry 31*, 11405–11412.
Pappa, H. S., & Cass, A. E. (1993) *Eur. J. Biochem. 212*, 227–235.
Prusiner, S. B. (1991) *Science 252*, 1515–1522.
Prusiner, S. B., Groth, D. F., McKinley, M. P., Cochran, S. P., Bowman, K. A., & Kasper, K. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4606–4610.
Prusiner, S. B., Scott, M., Foster, D., Pan, K.-M., Groth, D. F., Mirenda, C., Torchia, M., Yang, S. L., Serban, D., & Carlson, G. A. (1990) *Cell 63*, 673–686.
Prusiner, S. B., Groth, D. G., Serban, A., Stahl, N., & Gabizon, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2793–2797.
Ramalingam, K., & Bello, J. (1993) *Biochemistry 32*, 253–259.
Selvaggini, C., De Gioia, L., Cantu, L., Ghibaudi, E., Diomede, L., Passerini, F., Forloni, G., Bugiani, O., Tagliavini, F., & Salmona, M. (1993) *Biochem. Biophys. Res. Commun. 194*, 1380–1386.
Serban, D., Taraboulos, A., DeArmond, S. J., & Prusiner, S. B. (1990) *Neurology 40*, 110–117.
Sklaviadis, T. K., Manuelidis, L., & Manuelidis, E. E. (1989) *J. Virol. 63*, 1212–1222.
Sklaviadis, T., Akowitz, A., Manuelidis, E. E., & Manuelidis, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5713–5717.
Snow, A. D., & Wight, T. N. (1989) *Neurobiol. Aging 10*, 481–497.
Snow, A. D., Kisilevsky, R., Willmer, J., Prusiner, S. B., & DeArmond, S. J. (1989) *Acta Neuropathol. 77*, 337–342.
Snow, A. D., Wight, T. N., Nochlin, D., Koike, Y., Kimata, K., DeArmond, S. J., & Prusiner, S. B. (1990) *Lab. Invest. 63*, 601–611.
Stahl, N., & Prusiner, S. B. (1991) *FASEB J. 5*, 2799–2807.
Stahl, N., Borchelt, D. R., Hsiao, K., & Prusiner, S. B. (1987) *Cell 51*, 229–240.
Stahl, N., Baldwin, M. A., Burlingame, A. L., & Prusiner, S. B. (1990) *Biochemistry 29*, 8879–8884.
Stahl, N., Baldwin, M. A., & Prusiner, S. B. (1991) *Cell Biol. Int. Rep. 15*, 853–862.
Stahl, N., Baldwin, M. A., Teplow, D. B., Hood, L., Gibson, B. W., Burlingame, A. L., & Prusiner, S. B. (1993) *Biochemistry 32*, 1991–2002.
Staniforth, R. A., Burston, S. G., Smith, C. J., Jackson, G. S., Badcoe, I. G., Atkinson, T., Holbrook, J. J., & Clarke, A. R. (1993) *Biochemistry 32*, 3842–3851.
Taraboulos, A., Rogers, M., Borchelt, D. R., McKinley, M. P., Scott, M., Serban, D., & Prusiner, S. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8262–8266.
Touati, A., Creuzenet, C., Chobert, J. M., Dufour, E., & Haertle, T. (1992) *J. Protein Chem. 11*, 613–621.
Wang, D. C., Taraschi, T. F., Rubin, E., & Janes, N. (1993) *Biochim. Biophys. Acta 1145*, 141–148.
Weissmann, C. (1991a) *Nature 349*, 569–571.
Weissmann, C. (1991b) *Nature 352*, 679–683.
Zerovnik, E., Jerala, R., Kroon Zitko, L., Pain, R. H., & Turk, V. (1992) *J. Biol. Chem. 267*, 9041–9046.

Articles

Molecular Characteristics of the Major Scrapie Prion Protein†

Room temperature enzyme digestion

David C. Bolton, Michael P. McKinley, and Stanley B. Prusiner*

ABSTRACT: A major protein was identified that purifies with the scrapie agent extracted from infected hamster brains. The protein, designated PrP 27–30, was differentiated from other proteins in purified fractions containing the scrapie agent by its microheterogeneity (M_r 27 000–30 000) and its unusual resistance to protease digestion. PrP 27–30 was found in all fractions enriched for scrapie prions by discontinuous sucrose gradient sedimentation or sodium dodecyl sarcosinate–agarose gel electrophoresis. It is unlikely that PrP 27–30 is a pathologic product because it was found in fractions isolated from the brains of hamsters sacrificed prior to the appearance of histopathology. If PrP 27–30 is present in normal brain, its concentration must be 100-fold lower than that found in equivalent fractions from scrapie-infected hamsters. Three protease-resistant proteins similar to PrP 27–30 were found in fractions obtained by discontinuous sucrose gradient sedimentation of scrapie-infected mouse brain. These proteins were not evident in corresponding fractions prepared from normal mouse brain. One-dimensional peptide maps comparing PrP 27–30 and normal hamster brain proteins of similar molecular weight demonstrated that PrP 27–30 has a primary structure which is distinct from these normal proteins. Heating substantially purified scrapie fractions to 100 °C in sodium dodecyl sulfate inactivated the prion and rendered PrP 27–30 susceptible to protease digestion. Though the scrapie agent appears to be hydrophobic, PrP 27–30 remained in the aqueous phase after extraction with organic solvents, indicating that it is probably not a proteolipid. PrP 27–30 is the first structural component of the scrapie prion to be identified.

The structure and biochemical composition of the scrapie agent have not been established despite intensive research efforts for more than 2 decades. The scrapie agent causes a slow, degenerative neurological disease of sheep and goats in which the clinical and pathologic features closely resemble those of two human diseases, kuru and Creutzfeldt–Jakob disease (CJD) (Gajdusek, 1977; Hadlow et al., 1980). The etiologic agents of these three diseases exhibit many unusual properties which distinguish them from other infectious pathogens (Alper et al., 1966, 1967; Hunter, 1972; Gajdusek, 1977; Diener et al., 1982; Prusiner, 1982). The unusual properties and inferred presence of a protein within the scrapie agent led to introduction of the term "prion" to describe and identify this group of infectious pathogens (Prusiner, 1982). Though most of the data characterizing the biochemical properties of these pathogens have been obtained from studies of the scrapie agent, evidence to date indicates that it is prototypic of the entire group (Gajdusek, 1977; Hadlow et al., 1980; Prusiner, 1982).

Although early studies suggested that a protein component might be required for expression of scrapie infectivity (Hunter et al., 1969; Millson et al., 1976; Cho, 1980), these results were inconclusive. Convincing evidence that the scrapie agent contains a protein was obtained following development of improved purification methods. In particular, inactivation of the scrapie agent after protease digestion and reversible chemical modification with diethyl pyrocarbonate (DEP)[1] was demonstrated only with substantially purified preparations (Prusiner et al., 1981; McKinley et al., 1981). Although no specific protein component was identified, the results presented in those studies and later confirmed by others (Lax et al., 1983) demonstrated the requirement for a functional protein within the scrapie agent.

Improvements in the methodology for purifying the scrapie agent led to the identification of a unique protein (Bolton et al., 1982; Prusiner et al., 1982a). The protein exhibited size microheterogeneity upon electrophoresis in SDS–polyacrylamide gels, showing an apparent relative molecular weight (M_r) of 27 000–30 000, and was unusually resistant to digestion by proteinase K under nondenaturing conditions (Bolton et al., 1982). The close association of this protein with the scrapie prion suggested, but did not establish, that it was a structural component.

Subsequent data indicated that the protein, designated PrP 27–30, is a component of the prion (McKinley et al., 1983). In extensively purified fractions, the concentration of PrP 27–30 correlated with the titer of the scrapie agent over a 10 000-fold range. In addition, prolonged digestion of these fractions with proteinase K resulted in concomitant decreases in the concentration of PrP 27–30 and scrapie agent titer. Similar digestions using trypsin or SV-8 protease degraded most other proteins but did not alter the concentration of PrP 27–30 or the prion titer. No other protein exhibited these characteristics.

In this paper, we present some biological and biochemical characteristics of PrP 27–30. The data presented here in

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[1] Abbreviations: DEP, diethyl pyrocarbonate; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); Sarkosyl, sodium dodecyl sarcosinate; SV-8, *Staphylococcus aureus* V-8; Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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conjunction with other evidence (McKinley et al., 1983) support the hypothesis that PrP 27–30 is a component of the scrapie prion.

Experimental Procedures

Materials. All chemicals were of the highest grades commercially available. Sarkosyl, sodium cholate, DOC, Triton X-100, PEG, and micrococcal nuclease were purchased from Sigma. Enzyme-grade ammonium sulfate and sucrose were purchased from Schwarz/Mann. Proteinase K was purchased from Merck. SV-8 protease and α-chymotrypsin were obtained from Miles Laboratories. SDS was obtained from BDH Chemicals. Acrylamide and *N,N'*-methylenebis(acrylamide) were purchased from Bio-Rad Laboratories. $Na^{125}I$ was obtained from Amersham. *N*-Succinimidyl 3-(4-hydroxyphenyl)propionate was purchased from Calbiochem.

Source of Scrapie Prions and Bioassay. A hamster-adapted isolate of the scrapie agent was passaged and prepared as described (Prusiner et al., 1980a, 1982a). The Chandler isolate of the mouse-adapted scrapie agent was passaged as described (Chandler, 1961; Prusiner et al., 1977, 1978a,b) and, following an incubation period of 126–131 days, was isolated from infected brain homogenates by sedimentation in discontinuous sucrose gradients (Prusiner et al., 1982a). Scrapie agent titers were determined by measuring the incubation time intervals from inoculation to the onset of neurological illness and to death (Prusiner et al., 1980a, 1982b).

Purification of Scrapie Prions. (*A*) *Sedimentation through Discontinuous Sucrose Gradients.* The procedure was described in detail by Prusiner et al. (1982a). Briefly, homogenates of infected brain (10% w/v) were prepared in 320 mM sucrose and clarified by two low-speed centrifugations. Triton X-100 and DOC were added, and the scrapie agent was precipitated from the suspension upon addition of PEG. The resuspended pellet was sequentially digested with micrococcal nuclease (12.5 units/mL for 16 h) and proteinase K (100 μg/mL for 8 h). Sodium cholate was added, and the prions were removed from the suspension by precipitation with ammonium sulfate. Triton X-100 and SDS were added to the resuspended pellet, and the scrapie agent was sedimented through a 25% (w/v) sucrose solution onto a 60% sucrose cushion. Fraction 2 at the 25%/60% sucrose interface of this gradient contained scrapie prions purified approximately 1000-fold with respect to protein in the homogenate (Prusiner et al., 1982a).

(*B*) *Electrophoresis in Sarkosyl–Agarose Gels.* The procedure was described in detail by Prusiner et al. (1981). Briefly, homogenates of infected brain (10% w/v) were prepared as described above and clarified by two low-speed centrifugations. Prions were removed from the suspension by sedimentation to a pellet in a zonal rotor (32000 rpm for 16 h). The resuspended pellet was made 0.5% with DOC, and the scrapie agent was again sedimented to a pellet (50000 rpm for 2 h). The resuspended pellet was sequentially digested with micrococcal nuclease and proteinase K as described above. Sodium cholate was added and the scrapie agent precipitated from the digested suspension by addition of ammonium sulfate. Following dialysis to remove residual ammonium sulfate, the scrapie agent was electrophoresed into an 0.8% agarose gel having a 2-cm path length. The scrapie agent was removed from this gel by electroelution or by pulverizing the gel with a polytron device. The prions in these fractions (E_6) were purified approximately 100-fold with respect to protein in the homogenate (Prusiner et al., 1981).

Purification of Normal Brain Protein Fractions. Fractions corresponding to those containing the substantially purified scrapie agent were prepared by the two procedures described above. The animals used were uninoculated or inoculated with normal hamster brain homogenates analogous to the scrapie-containing inocula. The control animals were held for comparable periods of time as specified in Table II.

Radioiodination of Proteins. Protein fractions were concentrated by precipitation with SDS and quinine hemisulfate (Durbin & Manning, 1984) and the pellets washed with 80% acetone. For Bolton–Hunter labeling, the protein pellets were resuspended in 0.1 M sodium borate and 0.1% SDS (pH 8.5) and radioiodinated with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate as described (Bolton & Hunter, 1973). The radioiodination reaction was terminated by addition of 0.5 mL of 0.2 M glycine in 0.1 M sodium borate (pH 7.5). The iodinated proteins were removed from residual glycine and radioiodinated glycine by reprecipitation with SDS and quinine hemisulfate.

Labeling of the precipitated proteins resuspended in 50 mM sodium phosphate and 0.1% SDS (pH 7.5) with $Na^{125}I$ was performed by using chloramine-T according to a modification of the procedure described by Hunter & Greenwood (1962). Radioiodination of the concentrated proteins resuspended in 30 mM sodium phosphate was performed by using Iodobeads as described (Markwell, 1982). The efficiency of labeling could be enhanced by addition of 2% SDS and 8.0 M urea to the buffer, though proteins were undoubtedly denatured under those conditions. In some cases, the labeled proteins were reprecipitated by using SDS–quinine hemisulfate prior to separation by electrophoresis.

Polyacrylamide Gel Electrophoresis. Radiolabeled proteins in electrophoresis sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue, pH 6.8) were heated to 100 °C for 2 min. The proteins were separated by electrophoresis through vertical slab polyacrylamide gels using the discontinuous pH buffer system described by Laemmli (1970). The gels were stained and destained, and autoradiographic exposures were made as described previously (Bolton et al., 1982; Prusiner et al., 1982a).

One-Dimensional Peptide Mapping. Radioiodinated proteins were pretreated with proteinase K (100 μg/mL) in 100 mM Tris-HCl (pH 7.4) or with Tris buffer alone for 30 min. The digestions were terminated upon addition of an equal volume of 2× concentrated electrophoresis sample buffer and boiling at 100 °C for 2 min. The denatured proteins were separated by SDS–polyacrylamide gel electrophoresis as described above. The region containing PrP 27–30 (migration range of approximately 27000–30000 daltons), or the corresponding region of gels containing normal brain proteins, was identified by using an autoradiograph of the gel and cut from the gel with a razor blade. The proteins were eluted from these gel fragments into sterile 62.5 mM Tris-HCl buffer (pH 6.8) containing 0.2% SDS at room temperature over 24–48 h. The eluted proteins were concentrated by precipitation with quinine hemisulfate and the pellets washed with 80% acetone. The pellets were resuspended in digestion buffer containing 0.125 M Tris-HCl, 0.5% SDS, 10% glycerol, and 0.001% bromphenol blue (pH 6.8) and heated to 100 °C for 2 min. Aliquots of the denatured proteins were treated with digestion buffer only or with SV-8 protease or α-chymotrypsin at final concentrations of 133 μg/mL (Cleveland et al., 1977). The digestions were conducted at 37 °C for the time periods indicated in the legends to Figures 6 and 7. The peptide fragments were separated on 20% polyacrylamide gels, and autoradiography was performed as described (Bolton et al., 1982; Prusiner et al., 1982a).

Concentrating PrP 27–30. Six methods for concentrating PrP 27–30 from 200-μL aliquots of scrapie sucrose gradient fractions were studied. These were the following: (1) The suspension was made 0.2% with SDS, and the protein–SDS complexes were precipitated upon addition of 40 μL of 0.1 M quinine hemisulfate and 0.1 N HCl. The suspension was diluted with 1.0 mL of double-distilled water and cooled to 0 °C for 20 min prior to sedimentation at room temperature for 20 min in a microcentrifuge. The pellets were vigorously washed with 80% acetone and repelleted as before (Durbin & Manning, 1984). (2) The suspension was made 0.015% with DOC and the protein precipitated upon addition of 40 μL of 50% Cl_3CCOOH. The suspension was diluted with 1.0 mL of 10% Cl_3CCOOH and cooled to 0 °C for 20 min prior to sedimentation as described above. The pellets were vigorously washed with 80% acetone and repelleted as before. (3) The suspension was made 80% by volume with acetone and cooled to 0 °C for 20 min. The precipitated proteins were sedimented as described above. (4) The suspension was made 80% by volume with ethanol and treated as described above for acetone. (5) The suspension was made 80% by volume with methanol and treated as described above for acetone. (6) The suspension was diluted with 4 volumes of double-distilled water and treated as described above for acetone. The precipitated proteins were radioiodinated with the Bolton–Hunter reagent and analyzed by SDS–polyacrylamide gel electrophoresis.

Organic Solvent Extractions. Three different organic solvent combinations were used in an attempt to extract PrP 27–30 from the aqueous phase. To each 200-μL aliquot of scrapie sucrose gradient fraction 2 was added 800 μL of chloroform–methanol (2:1), chloroform, or toluene–ethyl acetate (1:1). The two-phase systems were vigorously mixed and cooled to 0 °C for 20 min prior to separation of the phases by centrifugation in a microcentrifuge. The control sample consisted of a 200-μL aliquot of fraction 2 which was diluted with 800 μL of double-distilled water and treated in an identical manner. The phases were collected separately, and the pellets were resuspended in 20 μL of 0.1 M sodium borate (pH 8.5) containing 0.1% SDS. Proteins in the aqueous phase were concentrated by precipitation with SDS and quinine hemisulfate as described above. Proteins in the organic phase were concentrated by evaporation to dryness with nitrogen. The concentrated proteins were radioiodinated and analyzed by SDS–polyacrylamide gel electrophoresis as described above.

Results

Detection of PrP 27–30. Two unusual properties of PrP 27–30 served to distinguish it from other proteins obtained from scrapie-infected or normal hamster brain (Bolton et al., 1982; Prusiner et al., 1982a). First, PrP 27–30 exhibited size microheterogeneity which was readily evident upon separation by SDS–polyacrylamide gel electrophoresis. Second, PrP 27–30 showed resistance to digestion by a variety of proteases under nondenaturing conditions. In this report, we used these two properties as the criteria for establishing the presence and absence of PrP 27–30 in fractions prepared from scrapie and normal hamster brains, respectively.

Figure 1 illustrates the application of these criteria for detecting PrP 27–30. Protease digestion for 30 min under nondenaturing conditions hydrolyzed all proteins in normal brain fractions. Under these conditions, cellular proteins in scrapie fractions also were hydrolyzed, but PrP 27–30 was left intact (Figure 1B). Thus, the protease resistance of PrP 27–30 did not result from the presence of a protease inhibitor in the scrapie fraction. The protein band which migrated slightly ahead of PrP 27–30 in these gels is probably a cleavage product

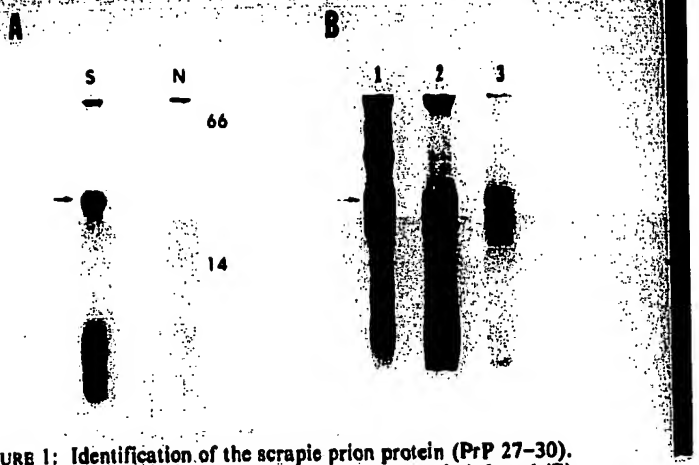

FIGURE 1: Identification of the scrapie prion protein (PrP 27–30). (A) Aliquots of sucrose gradient fraction 2 from scrapie-infected (S) or normal (N) hamster brain were concentrated 10-fold and radiolabeled with [^{14}C]DEP (Bolton et al., 1982). The radiolabeled proteins were resuspended in 10 mM Tris-HCl and 0.2% Sarkosyl (pH 7.4) and digested with proteinase K (100 μg/mL) for 30 min prior to electrophoresis in SDS–polyacrylamide gels. The autoradiograph was exposed for 720 h. The arrow indicates the position of PrP 27–30. (B) Aliquots of scrapie sucrose gradient fraction 2 were concentrated and radioiodinated with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate. The radioiodinated fractions were incubated at 37 °C for 30 min in 10 mM Tris-HCl and 0.2% Sarkosyl (pH 7.4) alone (lane 1) or containing 500 μg/mL SV-8 protease (lane 2) or 100 μg/mL proteinase K (lane 3) prior to electrophoresis in SDS–polyacrylamide gels. The autoradiograph was exposed for 24 h. The arrow indicates the position of PrP 27–30.

Table I: Scrapie Prions Were Unaltered by Iodination or Protease Digestion

(A) Chemical Modification by ^{127}I Incorporation	log titer (ID_{50} units/mL ± SE)	
procedure	before labeling	after labeling
Bolton–Hunter	8.3 ± 0.2	8.2 ± 0.2
chloramine-T	10.2 ± 0.1	10.1 ± 0.1

(B) Protease Digestion of ^{127}I-Labeled Bolton–Hunter-Labeled Fractions	
protease	log titer (ID_{50} units/mL ± SE)
none	8.1 ± 0.2
SV-8	8.4 ± 0.2
proteinase K	8.7 ± 0.2

of PrP 27–30. Possibly, this protein was generated during purification since prolonged digestion with proteinase K was utilized. The lower molecular weight protein has been observed frequently in purified fractions prior to additional protease treatment and has not been seen uniformly following secondary proteinase K digestion of the purified fractions.

Several methods for detecting PrP 27–30 have been employed. The most sensitive methods involved chemically labeling the protein with ^{125}I. In general, the chloramine-T method was slightly more efficient than the Iodobead procedure, and both gave significantly greater incorporation of radioiodine than the Bolton–Hunter method. No change in the titer of samples iodinated by two of these methods was observed (Table IA). In addition, PrP 27–30 has been detected in polyacrylamide gels by two different silver staining techniques (Merril et al., 1981; Morrissey, 1981). Detection of PrP 27–30 by staining with Coomassie blue has rarely been successful due to the low concentrations of PrP 27–30 available for electrophoretic analysis.

Denaturation of PrP 27–30 and Reduction of Prion Titer. Digestion of substantially purified fractions with proteinase K under the conditions described above did not alter the prion

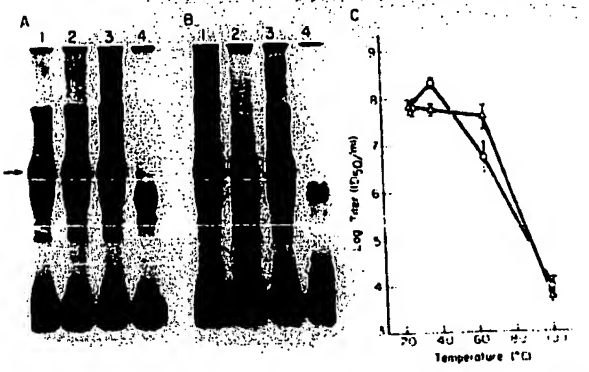

FIGURE 2: PrP 27–30 is denatured under conditions which reduce infectivity. Sucrose gradient purified scrapie suspensions were radioiodinated by using the Iodobead procedure and concentrated by sedimentation to a pellet in a microcentrifuge. The pellets were resuspended in electrophoresis sample buffer containing (A) 0.25% SDS and 1.25% 2-mercaptoethanol or (B) 1.25% SDS and 1.25% 2-mercaptoethanol. Ten-microliter aliquots of each suspension were heated to 25, 37, 65, and 100 °C for 2 min (lanes 1, 2, 3, and 4, respectively) and cooled to room temperature prior to the addition of 1 μL of proteinase K (1 mg/mL). The digestion was terminated after 30 min by adding 1 μL of phenylmethanesulfonyl fluoride (1 mM) followed by 12 μL of 2× concentrated electrophoresis sample buffer. Immediately, the samples were boiled for 5 min and the proteins separated on a 15% acrylamide gel. The autoradiograph was exposed for 17 h. The arrow indicates the position of PrP 27–30. (C) Sucrose gradient purified scrapie suspension was concentrated and the pellet resuspended in electrophoresis sample buffer containing 0.25% SDS and 1.25% 2-mercaptoethanol (Δ) or 1.25% SDS and 1.25% 2-mercaptoethanol (O). Aliquots were heated for 2 min as indicated, then diluted 100-fold in phosphate-buffered saline containing 5% bovine serum albumin, frozen in dry ice, and stored at −70 °C prior to bioassay. The vertical bars indicate standard errors.

titer. Identical samples iodinated with *N*-succinimidyl 3-(4-hydroxy-5-[^{127}I]iodophenyl)propionate and digested with SV-8 protease or proteinase K showed no significant change in titer (Table IB).

PrP 27–30 remained resistant to digestion with proteinase K after being heated to 65 °C in 0.25% SDS and 1.25% 2-mercaptoethanol (Figure 2A) or in 1.25% SDS and 1.25% 2-mercaptoethanol (Figure 2B). However, heating fractions to 100 °C in SDS and 2-mercaptoethanol denatured PrP 27–30 and rendered it protease sensitive. Infectivity also was stable after heating to 65 °C but was reduced by a factor of $\sim 10^4$ after incubation at 100 °C (Figure 2C). Thus, PrP 27–30 was denatured under conditions which inactivated the prion.

PrP 27–30 in Sucrose Gradient Scrapie Fractions. We have detected PrP 27–30 in 28 preparations of the scrapie agent purified by sucrose gradient sedimentation (Table II). PrP 27–30 was uniformly found in fractions prepared from hamsters inoculated 47–83 days prior to sacrifice. In general, we have found that the concentration of PrP 27–30 in purified fractions from these preparations was useful in predicting the titer.

We have not detected PrP 27–30 in analogous fractions prepared from normal hamster brain by the sucrose gradient sedimentation protocol (Table II). Fractions obtained from brains of uninoculated hamsters ranging in age from weanlings to approximately 120 days were analyzed for the presence of PrP 27–30. In addition, we searched for PrP 27–30 in analogous fractions prepared from weanlings inoculated with a 10% suspension of normal hamster brain and held for comparable incubation periods. By the criteria established above, PrP 27–30 was not present in any normal brain fraction examined (Table II).

Table II: PrP 27–30 and the Scrapie Prion Copurify by Two Different Methods

inoculum[a]	sex[b]	no. of purified prepn	time from inoculation to sacrifice (days)	PrP[c]
		(A) Purification by Discontinuous Sucrose Gradient Sedimentation		
S	M	2	68, 70	+
S	F	4	68 (2),[d] 71, 76	+
S	M/F	22	47, 63, 64, 66 (2), 67, 68 (6), 69, 70 (3), 73, 77, 73–78, 75–77, 76–78, 69–83	+
none	M/F	5	0,[e] 41–90 (2), 57–65, 68–90	−
N	M	1	66	−
N_2	M/F	3	63, 73, 75	−
		(B) Purification by Sarkosyl–Agarose Gel Electrophoresis		
S	M	2	67 (2)	+
S	M/F	3	63 (2), 65	+
none	M/F	4	0[e] (4)	−

[a] Ten percent homogenate of scrapie-infected (S) or normal (N) brain. [b] Males (M), females (F), or males and females together (M/F). [c] Presence (+) or absence (−) of PrP 27–30 in purified fractions. [d] Number of preparations in parentheses if greater than 1. [e] Equivalent number of days from time of inoculation.

FIGURE 3: PrP 27–30 and the scrapie prion copurify by Sarkosyl–agarose gel electrophoresis. Purified fraction E_6 was obtained from preparations of scrapie-infected hamster brain (lane 1) or normal hamster brain (lane 2). Aliquots were concentrated, radioiodinated with the Bolton–Hunter reagent, and then digested with proteinase K as described in Figure 1. The autoradiograph was exposed for 72 h. The arrow indicates the position of PrP 27–30.

PrP 27–30 in Sarkosyl–Agarose Gel Electrophoresis Fractions. Our success in detecting PrP 27–30 in sucrose gradient preparations highly enriched for the scrapie agent prompted a search for the protein in fractions purified by another protocol. The final purification step used in that protocol, Sarkosyl–agarose gel electrophoresis, separated macromolecules on the basis of different physical principles than the sucrose gradient sedimentation procedure. Therefore, although the procedure was less efficient in concentrating and purifying the scrapie agent, the presence of PrP 27–30 in scrapie fractions prepared by that method would provide evidence against artifactual copurification.

PrP 27–30 was found in scrapie fractions from five different preparations purified by this method (Table II, Figure 3). The concentration of the scrapie agent in these fractions was 50–100-fold less than that generally found in sucrose gradient fractions and varied over a 20-fold range between the five fractions tested. In general, we found that the concentration of PrP 27–30 in each of these fractions corresponded with the titer. Analysis of equivalent fractions prepared from normal hamster brain by the Sarkosyl–agarose gel electrophoresis

FIGURE 4: Identification of PrP 27–30 in fractions prepared from brains of hamsters sacrificed 47 days after inoculation. Aliquots of fractions 2 and 15 from a sucrose gradient prepared from hamsters inoculated with the scrapie agent 47 days previously were radioiodinated with the Bolton–Hunter reagent. The samples were digested with proteinase K as described in Figure 1 for 30 min prior to denaturation in electrophoresis buffer at 100 °C for 2 min. The samples were analyzed by electrophoresis on a 15% polyacrylamide gel. The autoradiograph was exposed for 69 h. Lane 1, fraction 2, buffer only; lane 2, fraction 2, proteinase K; lane 3, fraction 15, buffer only; lane 4, fraction 15, proteinase K. The arrow indicates the position of PrP 27–30.

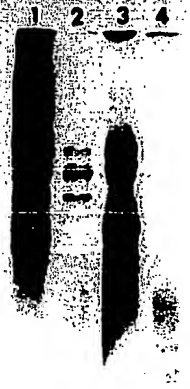

FIGURE 5: Protease-resistant proteins in fractions isolated from scrapie-infected mouse brain. Sucrose gradient fraction 2 was prepared from homogenates of mouse brain infected with the Chandler strain of the scrapie agent (lanes 1 and 2) c. homogenates of normal mouse brain (lanes 3 and 4). The proteins were concentrated and radioiodinated as described (Bolton et al., 1982) and then incubated for 30 min with 10 mM Tris-HCl and 0.2% Sarkosyl (pH 7.4) alone (lanes 1 and 3) or with this buffer containing 100 μg/mL proteinase K (lanes 2 and 4). The digestion was terminated by heating to 100 °C for 2 min after addition of an equal volume of 2× concentrated electrophoresis sample buffer, and the samples were separated by electrophoresis in a 15% polyacrylamide gel.

technique failed to show PrP 27–30 (Table II, Figure 3).

Identification of PrP 27–30 in Brains prior to Pathology. We looked for PrP 27–30 in the brains of hamsters at 47 days postinoculation because it would establish the presence of the protein in the brain prior to the appearance of histopathology (Baringer et al., 1983). By use of the discontinuous sucrose gradient method, the scrapie agent was substantially purified from a brain homogenate obtained from hamsters sacrificed 47 days postinoculation. Analysis of the fractionated sucrose gradient suggested that PrP 27–30 was present in those fractions (F-2 and F-15) which normally contain the highest concentrations of the scrapie agent when purified from hamsters sacrificed 65 days postinoculation (Bolton et al., 1982; Prusiner et al., 1982a). Treatment of these two fractions with proteinase K demonstrated that the protease-resistant protein, PrP 27–30, was present (Figure 4). Bioassay of the gradient fractions confirmed these findings. Fractions 2 and 15 were found to contain the highest titers, $10^{8.0}$ and $10^{7.9}$ ID_{50}/mL, respectively.

Protease-Resistant Proteins in Scrapie-Infected Mouse Brains. Three proteins resembling PrP 27–30 were found in fractions prepared from scrapie-infected mouse brain by discontinuous sucrose gradient sedimentation (Figure 5). These proteins were remarkably resistant to protease digestion, a characteristic of PrP 27–30. In addition, two of these proteins migrated with considerable size heterogeneity (M_r 29 200–32 000 and M_r 23 800–27 400) which is also characteristic of PrP 27–30. The third protein had an apparent molecular weight of 21 000. We could not detect these protease-resistant proteins in analogous fractions prepared from normal mouse brain (Figure 5, lane 4).

Search for PrP 27–30 in Normal Hamster Brain. The inability to detect PrP 27–30 in fractions purified from normal brain by either sucrose gradient sedimentation or Sarkosyl–agarose gel electrophoresis led us to define the limits of sensitivity of this analytical method. Aliquots of sucrose gradient fractions prepared from the brains of scrapie-infected and normal brain inoculated hamsters were radiolabeled with ^{125}I-labeled, Bolton–Hunter reagent. The radioiodinated samples were then digested with proteinase K (100 μg/mL) for 30 min at room temperature. Upon termination of the digestion, the samples were analyzed by SDS–polyacrylamide gel electrophoresis, and a series of autoradiographic exposures of these gels was used to determine the limits of detection. PrP 27–30 was visible on the autoradiograph of one proteinase K digested scrapie fraction after an exposure for only 0.5 h was seen in the autoradiograph of both proteinase K digested scrapie fractions after exposure for 6 h.

Identically purified normal brain fractions contained significant amounts of protein including numerous proteins with molecular weight values similar to that of PrP 27–30. However, proteinase K digestion of these fractions degraded virtually all of these proteins. PrP 27–30 was not detected in the autoradiograph of any of the proteinase K digested normal brain fractions following exposure for 532 h. We conclude that if PrP 27–30 is present in normal brain fractions its concentration must be <1% of that found in the equivalent scrapie fractions.

One-Dimensional Peptide Mapping of PrP 27–30 and Normal Brain Proteins. PrP 27–30 was not present in purified normal brain fractions as defined by the criteria of molecular size and resistance to proteolysis. However, several protease-sensitive proteins which migrated with apparent molecular weights comparable to that of PrP 27–30 were often found in normal brain preparations. We asked if these protease-sensitive proteins were related to PrP 27–30. The resistance of native PrP 27–30 to degradation by proteases could result from conformational alterations of an existing host protein. For example, an unusual secondary or tertiary structure of the polypeptide chain, or a quaternary structure such as aggregation, could confer protease resistance. One-dimensional peptide mapping studies comparing PrP 27–30 to normal brain proteins of similar molecular weight were conducted to resolve this question.

PrP 27–30 and the corresponding normal brain proteins were prepared from sucrose gradient fraction 2 and isolated by SDS–polyacrylamide gel electrophoresis following treatment with proteinase K (100 μg/mL) or Tris-HCl buffer alone. The denatured proteins were analzyed by a one-dimensional peptide mapping technique using SV-8 protease (Figure 6). Figure 6A shows the results of a control digestion

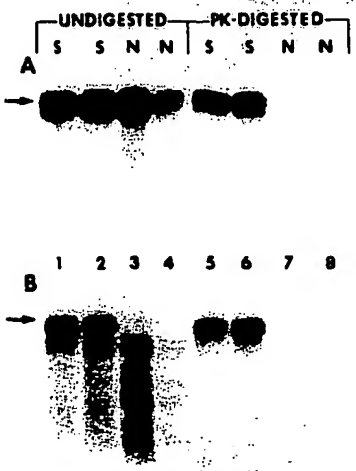

FIGURE 6: One-dimensional peptide mapping of PrP 27–30 and similar normal brain proteins. PrP 27–30 (S) and normal (N) brain proteins of similar molecular weight were isolated from sucrose gradient fraction 2 by SDS–polyacrylamide gel electrophoresis after incubation in Tris-HCl buffer alone ("UNDIGESTED") or proteinase K ("PK-DIGESTED"). The isolated proteins were denatured and incubated with digestion buffer alone (A) or with 133 μg/mL SV-8 protease (B) for 30 min. The digested samples were separated on 20% polyacrylamide gels. The arrows indicate a position 2 cm from the top of each gel.

in which the isolated proteins were electrophoresed again after incubation in digestion buffer alone. The proteins migrated as broad bands and showed little evidence of proteolytic degradation. As anticipated, almost no detectable protein remained in the normal brain sample digested with proteinase K prior to isolation (Figure 6A, lanes 7 and 8).

Digestion of the samples with SV-8 protease resulted in cleavage of a small peptide fragment from PrP 27–30, as shown by the small but significant increase in the electrophoretic mobility of the broad protein band (Figure 6B, lanes 1, 2, 5, and 6). SV-8 protease digestion of normal brain proteins produced discrete fragments migrating with a wide range of molecular weights. Most of these fragments could be found in the scrapie samples not pretreated with proteinase K (lanes 1 and 2), indicating contamination of these preparations with normal brain proteins. The slight amount of protein detected in the control digestion of proteinase K pretreated normal brain proteins (Figure 6A, lanes 7 and 8) was completely removed following digestion with SV-8 protease (Figure 6B, lanes 7 and 8). Similar results were obtained following digestion with α-chymotrypsin (data not shown). The molecular size and diversity of fragments generated by SV-8 protease and α-chymotrypsin digestion of the normal brain proteins distinguished them from PrP 27–30.

Kinetic studies confirmed that PrP 27–30 was cleaved by SV-8 protease. PrP 27–30 was isolated as described above and treated with SV-8 protease for periods ranging from 0 to 240 min. As shown in Figure 7A, a small peptide was cleaved from PrP 27–30, converting it to a faster migrating species. This process was evident as early as 5 min and was essentially complete within 60 min of digestion. The portion of PrP 27–30 remaining after this cleavage was stable to further digestion for 240 min.

SV-8 protease digestion of normal brain proteins of molecular weight similar to that of PrP 27–30 produced peptide fragments resembling those described in Figure 6. The fragments were not stable and were almost completely degraded within 180–240 min (Figure 7B). Peptide maps were produced from normal brain proteins predigested with proteinase K. No peptide fragments were found after an equivalent period of autoradiographic exposure of the gel (data not shown). However, prolonged exposure of the gel revealed patterns similar to those shown in Figure 7B (data not shown). These results clearly demonstrated primary structural differences between PrP 27–30 and normal brain proteins of similar molecular weight.

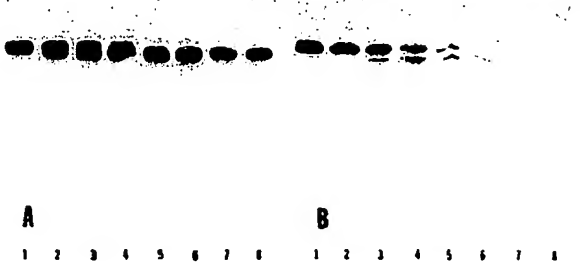

FIGURE 7: Kinetics of PrP 27–30 digestion by SV-8 protease. (A) PrP 27–30 was labeled with ^{125}I-labeled Bolton–Hunter reagent and digested with proteinase K as described in Figure 1 and then denatured by boiling in SDS and isolated by polyacrylamide gel electrophoresis. Denatured PrP 27–30 was then digested with SV-8 protease for 0, 5, 15, 30, 60, 120, 180, and 240 min as shown in lanes 1, 2, 3, 4, 5, 6, 7, and 8, respectively. The digestions were terminated upon addition of an equal volume of 2× concentrated electrophoresis sample buffer and heating to 100 °C for 2 min. The samples were electrophoresed in a 20% polyacrylamide gel. The autoradiograph was exposed for 22 h. (B) Normal brain proteins were labeled with ^{125}I-labeled Bolton–Hunter reagent and denatured by boiling in SDS. Proteins having molecular weight values of 27 000–30 000 were isolated from a polyacrylamide gel after electrophoresis. The denatured proteins were then digested with SV-8 protease, as described in (A). The autoradiograph was exposed for 22 h.

FIGURE 8: Concentration of PrP 27–30 from sucrose gradient fractions. Lane 1, SDS–quinine hemisulfate; lane 2, DOC–Cl_3CCOOH; lane 3, acetone; lane 4, methanol; lane 5, ethanol; lane 6, distilled water. Samples were analyzed by electrophoresis on a 15% polyacrylamide gel. The autoradiograph was exposed for 17 h. The arrow indicates the position of PrP 27–30.

Concentrating PrP 27–30. We found that the efficiency of radiolabeling and subsequent analysis of radiolabeled samples by SDS–polyacrylamide gel electrophoresis were enhanced by use of several concentration methods. These methods were employed to concentrate dilute protein suspensions and remove the protein(s) from buffers containing compounds which would inhibit the radiolabeling reaction. Following radiolabeling, the proteins were removed from unreacted reagents by a second precipitation. PrP 27–30 was found to be concentrated efficiently from sucrose gradient fractions by six different methods (Figure 8). The aggregated nature of the proteins in sucrose gradient fractions was shown by their sedimentation to a pellet by centrifugation at moderate speed in a microcentrifuge after dilution of sucrose with distilled water (lane 6).

Infectivity was preserved to varying degrees in samples concentrated by using these methods (Table III). In most

Table III: Concentration of Scrapie Prions

treatment	infectivity (log ID_{50}) recovered in each fraction: pellet	aqueous phase	sucrose phase	total infectivity recovered: log ID_{50}	%
DOC–Cl_3CCOOH[a]	1.3	2.8		2.8	<0.001
SDS–QS[b]	6.4	5.0		6.4	2
acetone	6.4	2.1	6.3	6.7	3
methanol	6.8	3.4		6.8	4
ethanol	7.5	4.3		7.5	20
H_2O	8.0	7.6		8.2	100

[a] DOC–Cl_3CCOOH = deoxycholate–trichloroacetic acid. [b] SDS–QS = sodium dodecyl sulfate–quinine hemisulfate.

cases, the majority of the recovered infectivity was found in the pellet. Infectivity was quantitatively recovered by sedimentation after dilution with distilled water alone. In this case, approximately 72% of the infectivity was found in the pellet.

Extraction of Sucrose Gradient Fractions with Organic Solvents. The size heterogeneity of PrP 27–30 and apparent hydrophobicity of the scrapie agent suggested that PrP 27–30 might be a proteolipid. Extraction of sucrose grudient fraction 2 with organic solvents demonstrated that PrP 27–30 was not soluble in these solvents. Extraction with chloroform–methanol (2:1), chloroform alone, or toluene–ethyl acetate (1:1) failed to partition PrP 27–30 into the organic phase (lanes 2, 4, and 6) to any significant extent. PrP 27–30 and most other proteins were found both in the pellet in and the aqueous phase following organic solvent extraction. Exclusion of PrP 27–30 from the organic solvent was not due to the density of the medium because toluene–ethyl acetate is less dense than water. Since PrP 27–30 was not extracted from the aqueous phase by organic solvents, it does not exhibit the properties characteristic of a proteolipid as originally defined by Folch & Lees (1951). PrP 27–30 might fulfill the criteria for proteolipids recently suggested by Schlesinger (1981), but this determination awaits the demonstration of fatty acid residues covalently bound to the protein.

Discussion

The studies presented here and in two other communications (McKinley et al., 1983; Prusiner et al., 1983) support our hypothesis that PrP 27–30 is the major protein component of the scrapie agent (Bolton et al., 1982; Prusiner et al., 1982a). PrP 27–30 is the first structural component of the scrapie prion to be identified.

PrP 27–30 and the prion were found to copurify by two different methods. Different techniques were used in each protocol to concentrate the prions from clarified supernatants. In the sucrose gradient procedure, PEG was added to precipitate prions from a suspension containing 4% Triton X-100 and 2% DOC (Prusiner et al., 1982a). For the electrophoretic procedure, prions were sedimented from the suspension by prolonged centrifugation in a zonal rotor and then repelleted by centrifugation at high speed in a fixed-angle rotor after extraction with 0.5% DOC (Prusiner et al., 1981).

The final steps of the two purification protocols utilized either discontinuous sucrose gradient centrifugation or Sarkosyl–agarose gel electrophoresis. In the sucrose gradient procedure, advantage was taken of the apparent hydrophobic behavior of the prions by sedimenting them from a suspension containing 0.2% Sarkosyl, 2% Triton X-100, and 0.8% SDS into a sucrose layer containing no detergents. It is likely that the scrapie agent and contaminating normal brain proteins aggregated as they sedimented into the sucrose layer lacking detergents and these aggregates rapidly sedimented to the interface of the 25% and 60% sucrose layers (Prusiner et al., 1982a). This behavior would be similar to that of the (Mg^{2+} + Ca^{2+})-ATPase, a membrane protein which has been shown to aggregate when sedimented into sucrose gradients devoid of detergents (Warren et al., 1974a,b). In the electrophoresis protocol, the scrapie agent was separated from contaminants by electrophoresis into a porous agarose gel under nondenaturing conditions. Negatively charged, rapidly migrating molecules including <5% of the recoverable prions were electrophoresed through the gel and eluted at the anode. Those prions which migrated less rapidly and thus remained in the gel under these conditions were eluted after electrophoresis by mechanical or electrophoretic procedures (Prusiner et al., 1980b, 1981).

A final point regarding differences between these two purification protocols concerns the kinds of detergents used in each. In the electrophoresis procedure, only the anionic detergents Sarkosyl, sodium cholate, and DOC were used (Prusiner et al., 1981). The sucrose gradient sedimentation procedure employed, in addition to these, the nonionic detergent Triton X-100 and the anionic detergent SDS (Prusiner et al., 1982a). It is difficult to determine what effects, if any, these differences had on the behavior of prions during purification because the use of detergents in the purification of macromolecules is largely an empirical process.

Copurification of PrP 27–30 and the scrapie agent throughout these two procedures indicates that the protein and the prion exhibit similar physicochemical properties. This behavior would be expected if PrP 27–30 were a component of the scrapie agent but would be unlikely if it were a product of the disease process. The presence of PrP 27–30 in the brains of infected hamsters prior to appearance of histopathology provides additional evidence against PrP 27–30 being a pathologic product. While normal brain proteins do purify with the scrapie agent by these two procedures, the proteins do not exhibit the resistance to protease digestion exhibited by PrP 27–30 and the prion (McKinley et al., 1983). If PrP 27–30 is present in normal brain fractions, it must exist at a concentration less than 1% of that in scrapie fractions, i.e., beyond our limits of detection.

Substantially purified fractions purified from scrapie-infected mouse brains contain protease-resistant proteins which resemble PrP 27–30 in their molecular size; thus, these proteins exhibit physicochemical properties similar to those of the scrapie agent and PrP 27–30. We could not detect these proteins in equivalent fractions prepared from normal mouse brain. We do not know whether the two lower molecular weight proteins represent distinct species or cleavage products of the largest protein. Further studies will be required to establish whether or not any of these three protease-resistant proteins found in scrapie-infected mouse brain are structural components of the murine scrapie prion.

Digestion of sucrose gradient fractions with proteinase K for 30 min under nondenaturing conditions degraded virtually all proteins in normal brain fractions and left only PrP 27–30 intact in scrapie fractions. Under these same conditions of proteolytic digestion, no change in infectivity was observed. Conversely, denaturation of scrapie prions by boiling in SDS and 2-mercaptoethanol rendered PrP 27–30 susceptible to degradation by proteases and diminished the infectivity by a factor of 10^4. These data demonstrate a correlation between infectivity and the conformation of PrP 27–30, thus providing additional evidence for PrP 27–30 being the major protein component of the prion. In another report, we demonstrated

that prolonging the time of digestion with proteinase K to 30 h under nondenaturing conditions significantly reduced scrapie infectivity concomitant with degradation of PrP 27–30 (McKinley et al., 1983). In contrast, digestion of scrapie prions with the amino acid specific proteases trypsin and SV-8 protease under these conditions degraded most other proteins but had no effect on infectivity or PrP 27–30. These properties are consistent with the behavior predicted for a macromolecular component of the scrapie agent.

The observation that normal brain fractions contained protease-sensitive proteins with electrophoretic properties similar to PrP 27–30 suggested that PrP 27–30 might represent a modified form of one or more of these proteins. An unusual secondary or tertiary structure in otherwise identical polypeptides could influence their susceptibility to digestion by proteases (Hilz et al., 1975; Rossmann & Argos, 1981; Ott et al., 1982), as could aggregation to form a protected multimeric structure (Tanford, 1961; Holzer & Heinrich, 1980). However, these noncovalent modifications would not affect the digestion patterns produced by cleaving the denatured proteins with amino acid specific proteases (Cleveland et al., 1977). We have shown that PrP 27–30 has a primary structure that is unique from these normal brain proteins when compared by one-dimensional peptide mapping.

Chemical modification and radiolabeling studies have shown that native PrP 27–30 probably has Lys, Tyr, and His residues on its surface or in positions accessible to the solvent (McKinley et al., 1981; Bolton et al., 1982). Chemical labeling by the method of Bolton and Hunter provides an efficient means of incorporating radioiodine into α- and ϵ-amino groups (Bolton & Hunter, 1973). It is not uncommon for proteins modified by this reagent to retain their biological activity (Langone, 1980). We have used this reagent to label PrP 27–30 under conditions which did not affect infectivity. This property made it possible to demonstrate, through proteolytic digestions studies, a correlation between degradation of PrP 27–30 and reduction of infectivity (McKinley et al., 1983).

At this time, the evidence implicating PrP 27–30 as the major protein component of the scrapie prion seems convincing. PrP 27–30 purifies with the scrapie agent by two different techniques and appears in the brain during the early phase of infection when prion titers are high but prior to clinical and pathologic manifestations of the disease. PrP 27–30 has not been observed in any fraction purified from normal hamster brain. The amount of PrP 27–30 in extensively purified scrapie fractions correlates with the titer of prions (McKinley et al., 1983). Treatments which inactivate the scrapie agent, such as thermal denaturation, chemical inactivation with DEP (McKinley et al., 1981; Bolton et al., 1982), or prolonged digestion with proteinase K (McKinley et al., 1983), effect corresponding changes in PrP 27–30. Conversely, treatments which degrade other proteins present in sucrose gradient fractions, such as digestion with proteinase K for 30 min or prolonged digestion with trypsin or SV-8 protease (McKinley et al., 1983), affect neither PrP 27–30 nor the prion. In our most purified fractions of the scrapie agent, PrP 27–30 is the major protein and accounts for 70–85% of the total detectable protein (Prusiner et al., 1983). We are aware of no data which contradict the hypothesis that PrP 27–30 is a structural component of the scrapie prion.

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References

Alper, T., Haig, D. A., & Clarke, M. C. (1966) *Biochem. Biophys. Res. Commun. 22*, 278–284.

Alper, T., Cramp, W. A., Haig, D. A., & Clarke, M. C. (1967) *Nature (London) 214*, 764–766.

Baringer, J. R., Bowman, K. A., & Prusiner, S. B. (1983) *J. Neuropathol. Exp. Neurol. 42*, 539–547.

Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529–539.

Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1982) *Science (Washington, D.C.) 218*, 1309–1311.

Chandler, R. L. (1961) *Lancet 1*, 1378–1379.

Cho, H. J. (1980) *Intervirology 14*, 213–216.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Diener, T. O., McKinley, M. P., & Prusiner, S. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5220–5224.

Durbin, R. K., & Manning, J. S. (1984) *Virology* (in press).

Folch, J., & Lees, M. B. (1951) *J. Biol. Chem. 191*, 807–817.

Gajdusek, D. C. (1977) *Science (Washington, D.C.) 197*, 943–960.

Hadlow, W. J., Prusiner, S. B., Kennedy, R. C., & Race, R. E. (1980) *Ann. Neurol. 8*, 628–631.

Hilz, H., Wiegers, U., & Adamietz, P. (1975) *Eur. J. Biochem. 56*, 103–108.

Holzer, H., & Heinrich, P. C. (1980) *Annu. Rev. Biochem. 49*, 63–91.

Hunter, G. D. (1972) *J. Infect. Dis. 125*, 427–440.

Hunter, G. D., Gibbons, R. A., Kimberlin, R. H., & Millson, G. C. (1969) *J. Comp. Pathol. 79*, 101–108.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Langone, J. J. (1980) *Methods Enzymol. 70*, 221–247.

Lax, A. J., Millson, G. C., & Manning, E. J. (1983) *Res. Vet. Sci. 34*, 155–158.

Markwell, M. A. K. (1982) *Anal. Biochem. 125*, 427–432.

McKinley, M. P., Masiarz, F. R., & Prusiner, S. B. (1981) *Science (Washington, D.C.) 214*, 1259–1261.

McKinley, M. P., Bolton, D. C., & Prusiner, S. B. (1983) *Cell (Cambridge, Mass.) 35*, 57–62.

Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science (Washington, D.C.) 211*, 1437–1438.

Millson, G. C., Hunter, G. D., & Kimberlin, R. H. (1976) in *Slow Virus Diseases of Animals and Man* (Kimberlin, R. H., Ed.) pp 243–266, North-Holland Publishing Co., Amsterdam.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Ott, U., Odermatt, E., Engel, J., Furthmayr, H., & Timpl, R. (1982) *Eur. J. Biochem. 123*, 63–72.

Prusiner, S. B. (1982) *Science (Washington, D.C.) 216*, 136–144.

Prusiner, S. B., Hadlow, W. J., Eklund, C. M., & Race, R. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4656–4660.

Prusiner, S. B., Hadlow, W. J., Eklund, C. M., Race, R. E., & Cochran, S. P. (1978a) *Biochemistry 17*, 4987–4992.

Prusiner, S. B., Hadlow, W. J., Garfin, D. E., Cochran, S. P., Baringer, J. R., Race, R. E., & Eklund, C. M. (1978b) *Biochemistry 17*, 4993–4999.

Prusiner, S. B., Groth, D. F., Cochran, S. P., Masiarz, F. R., McKinley, M. P., & Martinez, H. M. (1980a) *Biochemistry 19*, 4883–4891.

Prusiner, S. B., Groth, D. F., Cochran, S. P., McKinley, M. P., & Masiarz, F. R. (1980b) *Biochemistry 19*, 4892–4898.

Prusiner, S. B., McKinley, M. P., Groth, D. F., Bowman, K. A., Mock, N. I., Cochran, S. P., & Masiarz, F. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6675–6679.

Prusiner, S. B., Bolton, D. C., Groth, D. F., Bowman, K. A., Cochran, S. P., & McKinley, M. P. (1982a) *Biochemistry 21*, 6942–6950.

Prusiner, S. B., Cochran, S. P., Groth, D. F., Downey, D. E., Bowman, K. A., & Martinez, H. M. (1982b) *Ann. Neurol. 11*, 353–358.

Prusiner, S. B., McKinley, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., & Glenner, G. G. (1983) *Cell (Cambridge, Mass.) 35*, 349–358.

Rossmann, M. G., & Argos, P. (1981) *Annu. Rev. Biochem. 50*, 497–532.

Schlesinger, M. J. (1981) *Annu. Rev. Biochem. 50*, 193–206.

Tanford, C. (1961) in *Physical Chemistry of Macromolecules*, pp 623–639, Wiley, New York.

Warren, G. B., Toon, P. A., Birdsall, N. J. M. Lee, A. G., & Metcalf, J. C. (1974a) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622–626.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalf, J. C. (1974b) *Biochemistry 13*, 5501–5507.

Volume Changes Associated with Cytochrome *c* Oxidase–Porphyrin Cytochrome *c* Equilibrium†

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ABSTRACT: The binding of a fluorescent derivative of cytochrome *c* to cytochrome *c* oxidase has been studied by use of pressure to perturb the equilibrium. $\Delta V°$ for the reaction

oxidase–porphyrin cytochrome $c \overset{K_d}{\rightleftharpoons}$ oxidase + porphyrin cytochrome *c*

was small and favored dissociation of the complex. Pressure-induced dissociation is to be expected if the major forces governing the equilibrium are electrostatic in nature. The dependence of log K_d on pressure is not linear but biphasic; high pressures lead to a decrease in K_d and association of the reactants. The latter fact indicates that the net compressibility of the complexes is greater than that of the free reactants, an unexpected result.

Cytochrome *c* oxidase (EC 1.9.3.1) catalyzes electron transfer from cytochrome *c* to oxygen. The oxidase is a mitochondrial protein, the role of which is to couple electron transport to the production of ATP. This is accomplished through the intermediate establishment of an electrochemical gradient across the mitochondrial membrane. There appears little doubt that the gradient is set up, at least in part, by the active pumping by the oxidase of protons from the mitochondrial matrix space to the cytosolic space (Wikstrom, 1977). The oxidase is an intrinsic membrane protein; it is large (M_r 200000) and contains at least seven subunits, two hemes, and two coppers. The structure of the oxidase has been admirably reviewed recently by Capaldi et al. (1983).

The interaction of cytochrome *c* oxidase with cytochrome *c* is similar to the interactions that cytochrome *c* shows with its other protein acceptors. A summary of the available information indicates that the interacting face of cytochrome *c* viewed from any of its acceptor molecules (cytochrome *c* reductase, mammalian sulfite oxidase, and cytochrome *c* peroxidase, as well as cytochrome *c* oxidase) consists of a positively charged surface with the center of the charge located near phenylalanine-82; Koppenol & Margoliash (1982) have postulated that the driving force for the orientation of the cytochrome *c* with respect to its partners is the large dipole moment that is associated with the sum total of positive and negative charge on the cytochrome *c*. The acceptor molecules have been postulated to have a complementary surface as part of their interaction domains. In support of this statement, all of the acceptor molecules show similar ionic strength dependencies (Wainio et al., 1960; Davies et al., 1964; Nicholls, 1964), similar interactions with modified cytochromes *c* or the cytochromes *c* of different species (Errede & Kamen, 1978; Davis et al., 1972; Brautigan et al., 1978; Ferguson-Miller et al., 1978; Smith et al., 1980, 1981), similar reaction rate constants (Errede & Kamen, 1978; Smith et al., 1981), and similar tendencies to form tight binding complexes (Yonetani & Ray, 1965; Nicholls, 1964; Mochan & Nicholls, 1972; Yu et al., 1975; Ferguson-Miller et al., 1976). In the case of the peroxidase, where a high-resolution three-dimensional structure is known, a negatively charged surface (Poulos & Kraut, 1980), complimentary to the positively charged surface of the cytochrone *c* (Swanson et al., 1977), has been located. In the case of the oxidase, it is likely that some of the negative charge on the oxidase comes from the cardiolipin that is normally associated with the protein (Erecinska et al., 1980).

One consequence of the binding of cytochrome *c* by the oxidase is that spaces between them are eliminated:

$$n\mathrm{H_2O} + \text{enzyme–cytochrome } c \rightleftharpoons \text{enzyme–}p\mathrm{H_2O} + \text{cytochrome } c\text{–}m\mathrm{H_2O} \quad (1)$$

The total volume occupied by the reactants[1] of eq 1 may be greater or less than that of the products; $\Delta V°$ for the reaction may be either positive or negative but is unlikely to be zero. As such, the equilibrium of (1) can be perturbed by pressure. From a study of the pressure dependence, one can deduce how

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[1] Reactants or products, as used here, must be taken to mean the sum of all the interacting species on either side of eq 1. This includes interactions of the oxidase and porphyrin cytochrome *c* with solvent, detergent, and other components of the buffer.

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